

THE INFLUENCE OF VARIOUS  
PHOTOPERIODS ON PLANT MORPHOGENESIS  
AND NITROGEN METABOLISM IN  
URTICA DIOICA (L) AND SPINACIA OLERACEA (L)

MARGARETA WELANDER

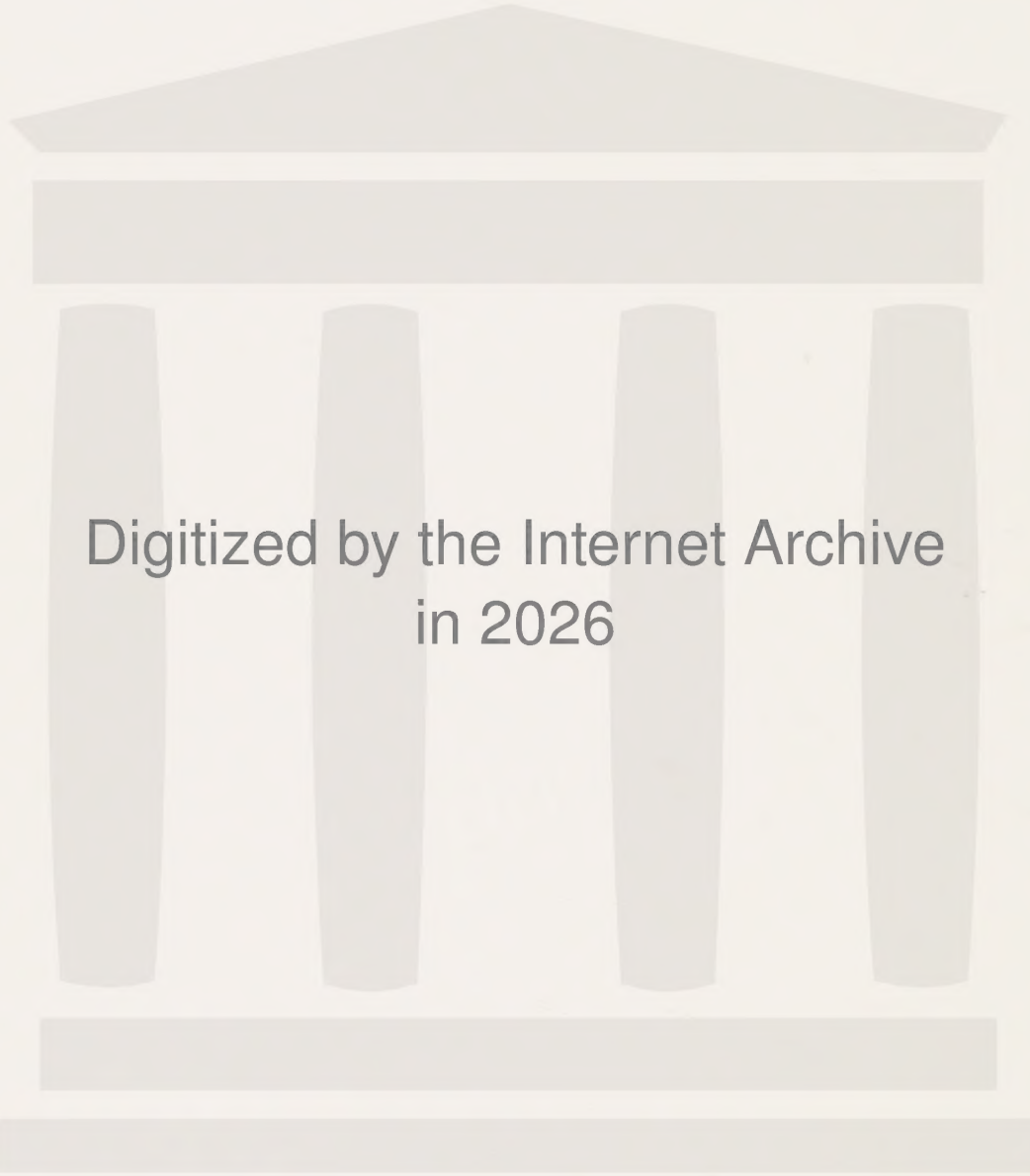
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PLANT MORPHOGENESIS AND NITROGEN METABOLISM IN  
URTICA DIOICA (L.) AND SPINACIA OLERACEA (L.)

By

Margareta Welander

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Doctoral dissertation

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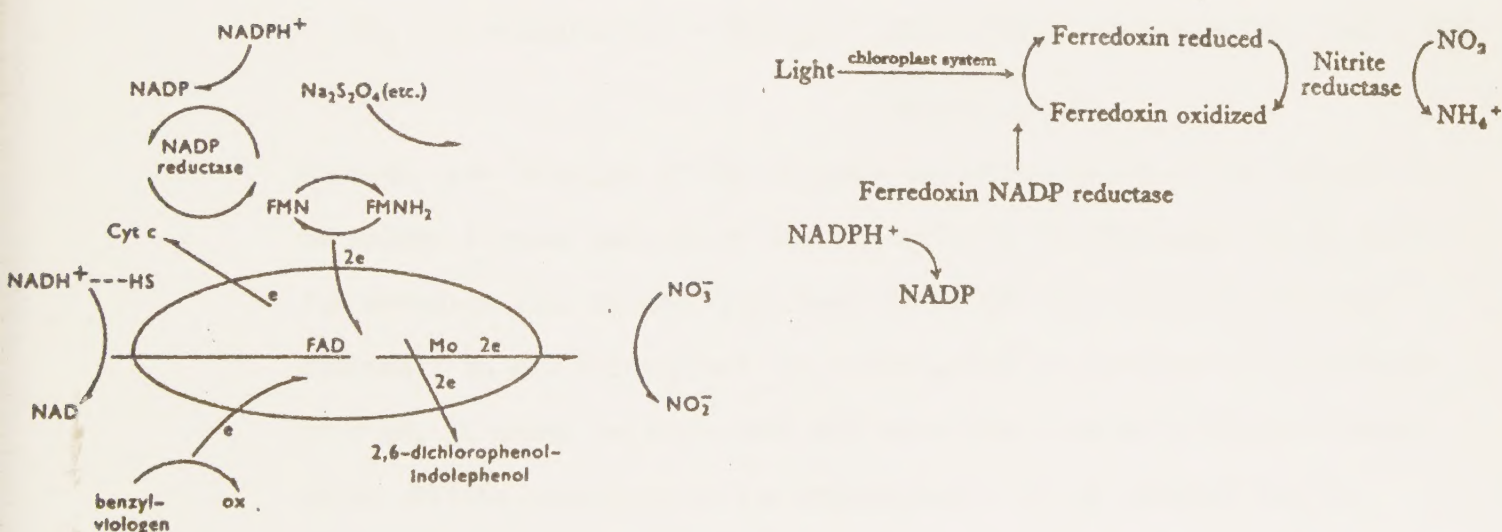
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## Introduction

Light has a major influence on plant morphogenesis. The growth habit of plants exposed to either long or short photoperiods is often profoundly different. The photoperiodic effect on plant morphogenesis has hitherto mostly been studied with particular respect to flower induction, dormancy induction and initiation of tuberization (Vince Prue 1975). Many morphogenetic responses are associated with changes in nitrogen metabolism (Mohr 1962). Alterations of the photoperiod and of light intensity can change the synthesis or the utilization of many nitrogenous compounds (Beevers 1976). Extending the daylength with photosynthetically active light will influence the rate of photosynthesis and thus alter the rate of formation of carbon skeletons available for amino acid biosynthesis. The close relationship between photosynthetic products and nitrogen metabolism was established by Steer (1973) and Warner and Kleinhof (1974). The enzymes responsible for nitrate reduction in higher plants are nitrate- and nitrite reductase.



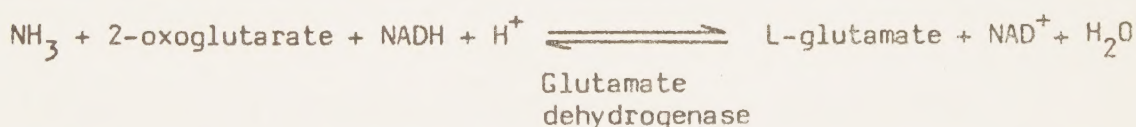
(From Beevers 1976)





Nitrate metabolism is very susceptible to a wide range of environmental conditions. It has earlier been demonstrated that light, mineral nutrition, plant age, and genetic composition influence the capacity of nitrate reduction (Nicholas et al. 1976, Weissman 1972, Liu and Hadley 1971, Wallace and Pate 1965, 1967). The control of nitrate reduction is probably brought about through a regulation of the enzyme nitrate reductase. There are several reasons to assume that nitrate reductase influences the regulation of the input of reduced nitrogen.

Nitrate reductase is the first enzyme in the assimilation pathway of nitrate and the rate limiting step. It is also known that nitrate reductase occurs in much lower quantities than other enzymes involved in the assimilation and reduction of nitrate (Lewis personal communications). Furthermore, nitrate reductase is substrate inducible and shows a high turnover rate (Zielke and Filner 1971). The end product in the reduction of nitrate is ammonium. In higher plants, glutamate dehydrogenase (GDH) has been considered until recently as one of the most important enzymes for the assimilation of ammonium ions into organic nitrogen compounds. It catalyzes the combination of 2-oxoglutarate with ammonia to yield glutamate and the reverse reaction of oxidative deamination of glutamate.



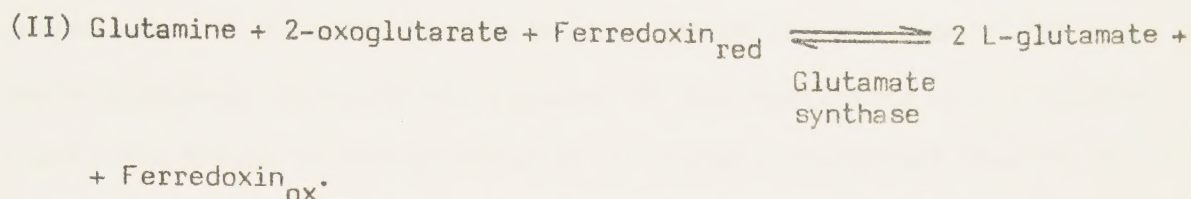
However, the function of GDH in ammonium assimilation is now thought to be rather limited because of its low affinity to ammonium. The  $K_m$  value for ammonium ions ranges from about 10 to 200 mM. Since the electron transport in the chloroplasts is curtailed at an ammonium concentration of 2 mM, it seems unlikely that GDH will take over as a primary enzyme after nitrite reduction in the chloroplasts. It is assumed that an alternative route for ammonium assimilation occurs on normal intracellular levels (Mifflin and Lea 1976). In the presence of glutamate, ammonium is incorporated as glutamine by glutamine synthetase (I). This enzyme





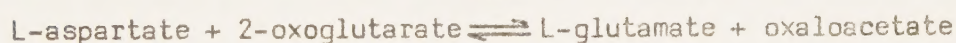
cooperates with glutamate synthase which transfers the amide group from glutamine to 2-oxoglutarate, producing two molecules of glutamate (II).

Glutamine  
synthetase

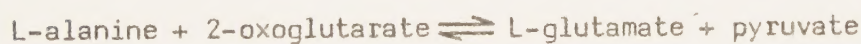


Higher plants contain many aminotransferases which play an essential part in the synthesis of amino acids used for protein biosynthesis. It has been suggested that aminotransferases provide an essential link between carbohydrate and amino acid metabolism (Walker 1964). In addition, the aminotransferases can form a shuttle system, transferring reducing substances between the cytosol and organelles, such as chloroplasts and peroxisomes (Rathnam and Edwards 1976). The two transaminases dealt with in this investigation are aspartate aminotransferase (glutamate-oxaloacetate transaminase, GOT) and alanine aminotransferase (glutamate-pyruvate transaminase, GPT). They catalyze the following reversible reactions:

GOT



GPT



It is widely recognized that changes in the enzyme level are an essential step for controlling plant growth development. The precise role of the individual enzymes, however, has not yet been established. Moreover, it seems as if the photomorphogenetic control of the enzyme level is quantitative rather than qualitative, bringing about a change in the level of an enzyme activity already present in measurable amounts.



The main purpose of this investigation has been to study the influence of photoperiodic variation on plant morphogenesis and protein production, and possible correlation between protein production and enzymes involved in nitrogen metabolism. Due to difficulties in obtaining enzyme activity in extracts from *Urtica* without the addition of PVP and mercaptoethanol, *Spinacia* was included in the experiments as a reference plant. The effect of mercaptoethanol on enzyme activity was studied in both *Urtica* and *Spinacia*. Since *Urtica* as well as *Spinacia* flower under LD conditions, I have tried to define the influence of the flowering process on enzyme activity.

The thesis is based on the following papers:

- I Welander, M. 1974. Enzyme activities in *Urtica dioica*: Effects of daylength and leaf age on glutamate dehydrogenase, aspartate aminotransferase and alanine aminotransferase. - *Physiol. Plant.* 30:192-199.
- II Welander, M. 1978. The effect of mercaptoethanol on the activity of enzymes of nitrogen metabolism in leaves from *Urtica dioica* and *Spinacia oleracea*. - *Physiol. Plant.* 43:242-246.
- III Welander, M. and Bergkvist, B. 1979. The influence of photoperiodic variation on plant morphogenesis and nitrogen metabolism in *Urtica dioica* and *Spinacia oleracea*. - Published in this dissertation.
- IV Welander, M. 1979. Investigation of plant morphogenesis and nitrogen metabolism during flower induction in *Spinacia oleracea*. - Published in this dissertation.





## Experimental methods

*Urtica dioica* L. (wild Sweden) and *Spinacia oleracea* L. (cv. Dominant Weibull and cv. Sperling's Monoppa) were used as plant material. The plants were grown in soil with admixture of a fertilizer or in silica sand irrigated daily with a nutrient solution. The cultivations were performed in climate chambers and the plants were subjected either to SD with 8 or 10 h light for *Spinacia* and 12 h light for *Urtica*, or to LD with 16 h light for *Spinacia* and 18 h light for *Urtica*. The *Urtica* plants were maintained at 22°C during the day and at 12°C during the night and the *Spinacia* plants were kept permanently at 22°C.

In all investigations, leaves of different age groups were used and in paper III roots were also included. After the plant material had been extracted, the proteins of the extracts were separated from low-molecular weight compounds on a Sephadex G25 column. The activities of GDH, GOT, GPT and NR were assayed spectrophotometrically. In some cases, the determination both of GOT and GDH activity and of unspecific proteins was performed by gel electrophoresis. NR activity was measured both in vivo and in vitro under specified circumstances. Water soluble proteins were measured according to Potty (1969). The amount of total protein obtained after TCA-extraction, the TCA-soluble non-protein fraction, and the content of ammonium were determined according to Kjeldahl, and so was the content of nitrate after reduction of nitrate to ammonium with Devarda's alloy. In order to characterize morphological changes in the apical meristem during flower induction shoot apices were sectioned with a freeze microtome.

Abbreviations: GDH: glutamate dehydrogenase; GOT: aspartate aminotransferase; GPT: alanine aminotransferase; NR: nitrate reductase; PVP: polyvinylpyrrolidone; TCA: trichloroacetic acid; LD: long day; SD: short day.





## Results

### I Enzyme activity in *Urtica dioica*: Effects of daylength and leaf age on glutamate dehydrogenase, aspartate aminotransferase and alanine aminotransferase.

Light energy increase from 336 to 504  $\text{W}\cdot\text{h}\cdot\text{m}^{-2}\cdot\text{day}^{-1}$  (the latter value as either LD or SD) had no effect on the total amount of protein per gram dry weight produced in the leaves. However, a further increase in light energy resulted in a higher amount of protein in young leaves and a lower amount of protein in old leaves. Young leaves contained more protein than old leaves, whereas the content of non-protein  $\alpha$ -amino nitrogen was higher in old leaves.

The activity of GDH is much higher in plants kept under LD conditions than in SD plants, irrespective of the amount of light energy. GDH activity also increased with leaf age. These results were confirmed both by photospectrometric measurements and by polyacrylamide gel electrophoresis. The activity of GPT responded to photoperiodic variations in the same way as did GDH. GPT activity also increased with leaf age. A high correlation between GDH and GPT activity was established, the correlation coefficient being 0.928. GOT activity did not respond to environmental changes. The electrophoretic separations of GOT revealed five isoenzymes in old leaves but only four in young leaves in both LD and SD.

The role of GDH in nitrogen metabolism is still disputed. The total amount of protein and the activity of GDH were not correlated to each other and responded differently to photoperiodic variations. From these results, it can be concluded that GDH activity is more correlated to catabolic processes occurring in the leaves.



## II The effect of mercaptoethanol on the activity of enzymes of nitrogen metabolism in leaves from *Urtica dioica* and *Spinacia oleracea*.

Mercaptoethanol interfered with the activities of GDH, GOT, GPT, and NR in leaves from *Urtica* and *Spinacia*. Leaf extract from *Urtica* immediately turned brown and no enzyme activity could be obtained without the addition of PVP. Isolation of GDH required, besides PVP, mercaptoethanol in the extraction medium in order to preserve GDH activity. Leaf extract from *Spinacia* remained green without the addition of protective agents, which indicates low activity of phenol oxidase and/or low content of phenols. In both plant species, the activity of GDH was stimulated by mercaptoethanol to a certain level. For *Urtica*, maximum activity was attained at a concentration of 0.28 M, and for *Spinacia* at a concentration of 0.14 M. GOT and GPT activity was positively affected by the same concentration of mercaptoethanol in leaf extract only from *Urtica*. In *Spinacia*, the inactivation of GOT and GPT activity with increasing concentration of mercaptoethanol is more pronounced for GOT than for GPT. In *Spinacia*, the apparent NR activity diminished markedly with increasing concentration of mercaptoethanol. No NR activity at all could be detected in *Urtica* at any concentration of mercaptoethanol. In the method used for assaying NR activity, the amount of nitrite produced was a measure of NR activity. It was shown that mercaptoethanol has the ability to reduce nitrite and thereby interfere with the determination method. On the basis of these results, it was suggested that mercaptoethanol included in extracts from *Urtica* has a dual effect: On the one hand, mercapthoethanol inhibits phenol oxidation and on the other hand it interferes with the investigated enzymes. This may explain why a higher concentration of mercaptoethanol is required to obtain maximum GDH activity in *Urtica* as compared with *Spinacia*.





The increased activities of GOT and GPT noted for *Urtica* were probably due to inhibition of phenol oxidation. It was difficult to draw any conclusions as to the effect of mercaptoethanol on NR activity, because mercaptoethanol itself interfered with the assay method for NR activity. The interference of mercaptoethanol with enzyme activity is thus dependent on the type of plant, the configuration of the enzyme, and the concentration of mercaptoethanol.



### III The influence of photoperiodic variation on plant morphogenesis and nitrogen metabolism in *Urtica dioica* and *Spinacia oleracea*.

Alteration of the photoperiod was found to change the growth habit in both *Urtica* and *Spinacia*. Both species flowered under LD conditions. In *Urtica*, the chlorophyll content in the leaves was measured and was found to be higher in LD than in SD. Extension of the daylength increased the fresh weight and the dry weight in both species. In *Urtica*, the amount of protein per plant was much higher in LD than in SD owing mainly to an increased protein content in stem plus leaf petioles and in roots. In *Spinacia*, all different plant parts produced more protein per g d.wt. in SD. Nevertheless, plants subjected to LD contained more protein than plants subjected to SD owing to the higher amount of dry matter produced in LD.

Most of the nitrate was reduced in the leaves in both *Urtica* and *Spinacia*. On the other hand, despite the low NR activity per g d.wt. in the roots, the contribution of the roots to the enzymatic potential should not be neglected. In *Urtica*, a positive correlation between total NR activity and total amount of proteins was obtained in leaves and roots. In *Spinacia*, such a correlation was found only in the roots. No consistent correlations were obtained from comparisons between the amount of protein per g d.wt. and NR activity per g d.wt. in the different plant parts.

The highest specific activity of GDH was obtained in roots in both *Urtica* and *Spinacia* under LD conditions. The specific activity of GDH in leaves from *Urtica* subjected to LD also increased. The specific activity of GPT in leaves from *Urtica* increased under LD conditions and was found to be well correlated with the chlorophyll content. This has earlier been demonstrated by Hedley and Stoddart (1971) and Thomas (1974). Minor changes were observed in the specific activity of GOT. In *Spinacia*, small changes in the specific activity of GPT in leaves of different age groups were observed with extended photoperiod. However, this did not





result in any changes in the total activity of the leaves. In *Urtica*, the specific activity of GOT and GPT in the roots did not change during the extended photoperiod. On the other hand, the total enzyme activity was higher during LD when the activity was expressed per root. In *Spinacia*, both the specific activity of GOT and GPT in the roots, and the total enzyme activity per root markedly increased under LD conditions.

#### IV Investigation of plant morphogenesis and nitrogen metabolism during flower induction in *Spinacia oleracea* (L.).

*Spinacia* plants were induced to flower in LD. The photoperiod was extended by low intensity light from red fluorescent tubes. Whether a plant was induced to flower could be seen from the development of the shoot apex. Plants subjected to LD increased in leaf area and leaf fresh weight but only minor changes in dry weight were observed.

A temporary increase in the content of total protein nitrogen was observed after two LD cycles in leaves from induced plants. This increase was probably due to the presence of membrane associated proteins since four LD cycles were required to obtain an increase in the content of soluble protein nitrogen. Polyacrylamide gel electrophoretic studies of soluble proteins from leaves of induced and non-induced plants did not reveal any differences in the stained protein pattern.

The content of nitrate in stem plus leaf petioles and in leaves increased under LD conditions. The increased amount of nitrate in leaves in LD did not increase specific NR activity. This indicates that there are different pools for nitrate storage and induction. No differences were observed in the specific activity of NR that could be related to either induction or evocation of flowering. Oscillations of the enzyme activity were observed in both induced and non-induced plants.

The specific activity of GPT temporarily decreased under LD conditions. This decrease occurred before the first visible change in shoot apex was recorded. The specific activity of GOT was similar



for plants in LD and SD until the LD plants had received 14 LD cycles. Thereafter, the GOT activity decreased more in LD. Besides these changes, the specific activity of GOT and GPT exhibited mutually similar oscillations in the leaves from plants subjected to LD or SD. The first differences in specific GDH activity between plants in LD and SD were observed after the induced plants had received 16 LD cycles. The oscillations in GDH activity did not show the same pattern as was found for the other enzymes. The oscillations in the activity of GOT, GPT and NR are probably due to different developmental stages of the leaves since the interval between the two peaks observed coincides with the interval for a plastochrone (Figure 1).



## Conclusions

From papers I and III it can be concluded that

1. The amount of fresh and dry matter as well as the protein content increase in *Urtica* and *Spinacia* plants under LD conditions.
2. In *Urtica*, the additional dry matter produced in LD is present only in stem plus leaf petioles and roots, whereas in *Spinacia* the extra dry matter is found in both leaves and roots.
3. In *Urtica*, a positive correlation between NR activity per plant part and the amount of proteins per plant part is obtained in leaves and roots. In *Spinacia*, such a correlation is found only in the roots. On the other hand, there are no consistent correlations between the amount of protein per g d. wt and NR activity per g d. wt in the different plant parts.
4. Most of the nitrate is reduced in the leaves in both *Urtica* and *Spinacia*. The highest specific and total activity of GDH is found in the roots under LD conditions. The specific activity of GDH in leaves also increases in LD and with leaf age, especially in *Urtica*. The results are consistent with the hypothesis that GDH is probably not involved in the assimilation of ammonia.
5. The total activity of GOT and GPT in roots from *Urtica* and *Spinacia*





increases under LD conditions. In *Spinacia*, the specific activity also increases in the roots, which is however not the case in *Urtica*. The specific activity of GPT increases in leaves from both species, whereas the specific activity of GOT is almost unaffected by photoperiodic variation.

From a comparison between *Spinacia* plants induced by low intensity light from red fluorescent tubes ( paper IV ) and plants induced by high intensity light from cool-white fluorescent tubes ( paper III ), it can be concluded that in the leaves :

1. The specific activity of nitrate reductase is not influenced by the flowering process. However, minor changes appear in the specific activity owing to photoperiodic variation.
2. The specific activity of alanine aminotransferase changes in LD before the first sign of flower induction appears, and may be involved in the induction process.
3. The specific activity of glutamate dehydrogenase and aspartate aminotransferase will not change until visible flower primordia have developed in LD. This indicates that these changes are a result of metabolic events caused by flowering and not by flower induction. No changes in the specific activity owing to photoperiodic extension are obtained with high intensity light.

Finally, it can be concluded from paper IV that



1. The oscillations in the activity of GOT, GPT and NR are probably due to different developmental stages of the leaves since the interval between the two peaks observed coincides with the interval for a plastochrone.





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## Enzyme Activities in *Urtica dioica*: Effects of Daylength and Leaf Age on Glutamate Dehydrogenase, Aspartate Aminotransferase and Alanine Aminotransferase

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### Abstract

Enzymes, important to protein synthesis, were investigated in young and old leaves of *Urtica dioica*. The plants, divided into two groups, were exposed to either 18-hour or 12-hour photoperiods. One group of plants from each photoperiodic regime was subjected to an irradiance of  $28 \text{ W} \times \text{m}^{-2}$ , and the other group of plants to  $42 \text{ W} \times \text{m}^{-2}$ . The enzymes investigated were glutamate dehydrogenase (GDH), aspartate aminotransferase (glutamate-oxaloacetate transaminase, GOT), and alanine aminotransferase (glutamate-pyruvate transaminase, GPT). GDH and GOT were determined by means of electrophoretic separation on polyacrylamide and spectrophotometric measurements. GPT was determined only by the latter method.

Plants exposed to 18-hour photoperiods showed much higher GDH activity than did those exposed to 12-hour photoperiods. The activity of GDH also increased with leaf age. Besides one uniform  $\text{NAD}^+$ -dependent GDH, two other  $\text{NAD}^+$ -independent enzymes, showing GDH activity, were identified on polyacrylamide gel electrophoresis. The distribution of NADH and  $\text{NAD}^+$ -dependent GDH activity between young and old leaves was similar under different growth conditions.

The activity of GOT was insensitive to environmental changes. The results regarding GPT indicate that this enzyme responded to different photoperiods in the same way as GDH. A correlation coefficient of 0.928 was obtained for the relationship between GDH and GPT activity.

### Introduction

The aim of the present investigation was to study the effect of daylength and leaf age on enzymes important to protein synthesis and on protein content. The enzymes studied are glutamate dehydrogenase (E.C. 1.4.1.2; GDH), aspartate aminotransferase (E.C. 2.6.1.1) also named glutamate-oxaloacetate transaminase (GOT) and alanine aminotrans-

ferase (E.C. 2.6.1.2) also named glutamate-pyruvate transaminase (GPT). GDH is of importance for the incorporation of inorganic nitrogen into amino acids. GOT and GPT are important in transaminating processes to convert and produce amino acids.

The effects on the protein metabolism due to environmental change and stress have been reported in several studies. In experiments on *Mentha piperita*, Steward *et al.* (1959) demonstrated that storage of soluble nitrogen in leaves was higher under short-day conditions than under long-day conditions. This was accompanied by relative suppression of protein synthesis. The main question is whether the decrease in the protein content is due to proteolytic activity or to a lower rate of synthesis. Some evidence showing that a lower rate of synthesis is of importance, was given for leaves from *Perilla frutescens* (Kannangara and Woolhouse 1968).

Changes in the transaminase activity in leaves of *Lolium temulentum*, exposed to either short or long days, were established by Hedley and Stoddart (1971, 1972).

Isoenzymes of GDH were investigated by Yue (1969) in several different plants. The number of isoenzymes and their electrophoretic mobility varied between the different plants studied. Pahlich and Joy (1971) stated that, in pea roots, the enzyme showed both NADH- and  $\text{NAD}^+$ -dependent GDH activity, although the ratio of these activities was not constant. However, the fundamental information about GDH effects on the regulatory mechanism in higher plants, is not sufficient.

**Abbreviations.** GDH: glutamic acid dehydrogenase; GOT: glutamate-oxaloacetate transaminase; GPT: glutamate-pyruvate transaminase;  $\text{NAD}^+$ , NADH: nicotinamide adenine dinucleotide oxidized and reduced; PVP: polyvinylpyrrolidone; TCA: trichloroacetic acid.



## Materials and Methods

### Plant material

*Urtica dioica* L. (wild, south of Sweden) was used in all the experiments. The seeds were sown in soil in boxes. Seedlings, 2–3 cm high, were planted in pots, 10 plants in each. Two weeks later, 10 g of Weibull's Enpekå Special fertilizer (12% N, 12%  $P_2O_5$ , 19%  $K_2O$  and micro-nutrients) was applied to each pot. Cultivation of the plants was performed in climate chambers under controlled conditions. The plants, divided into two groups, were exposed to either 18-hour (long day) or 12-hour (short day) photoperiods. One group of plants from each photoperiodic regime was subjected to the irradiance of  $28 W \times m^{-2}$  (10,000 lux), and the other group of plants to  $42 W \times m^{-2}$  (15,000 lux). The light energy was supplied from cool-white fluorescent lamps (F 48 PG17/CWC, General Electric). The day temperature was 22°C, and the night temperature 12°C. Daylengths and irradiances were chosen so that the same amount of light energy was received at  $28 W \times m^{-2}$  during 18 hours, as at  $42 W \times m^{-2}$  during 12 hours. The plants were watered daily with distilled water. Plants exposed to long days were harvested 30 days after planting, and plants exposed to short days 10 days later. The cultivations were performed twice.

### Sampling and extraction

The youngest (not fully expanded) leaves and the oldest ones (without deficiency symptoms) of each plant were used. The leaves were sampled simultaneously in order to avoid diurnal fluctuations in the enzyme level. All subsequent operations were carried out at 0–5°C.

Four grams fresh weight of leaf material was frozen in liquid nitrogen and pulverized with a mortar and pestle. The pulverized material was mixed with 3 g of Polyclar AT (polyvinylpyrrolidone PVP) and 20 ml buffer consisting of 0.1 M Tris-HCl, and 0.005 M mercaptoethanol at pH 7.5. The sample was ground with an Ultra-Turrax apparatus, which was rinsed with 10 ml buffer. The slurry was squeezed through double layers of cheese cloth. The filtrate was centrifuged at 1000 g for 10 minutes, at 0°C. The precipitate was discarded, and the resulting supernatant was centrifuged at 48,000 g for 30 minutes. The proteins of the supernatant were then purified from low-molecular weight components by passing through a Sephadex G25 column. A volume of 20 ml of the purified sample was concentrated against aquacide I for 90 minutes. To this protein extract was added dimethylsulfoxide to 25% and mercaptoethanol to a final concentration of 0.005 M. By the addition of dimethylsulfoxide and mercaptoethanol it was then possible to store the sample at –20°C without any loss of enzyme activity.

### Enzyme determinations

Qualitative and quantitative enzyme assays were carried out by means of polyacrylamide gel electrophoresis and spectrophotometric measurements. All enzyme determinations were repeated. Good correlation was found between electrophoretic and spectrophotometric measurements.

**Polyacrylamide gel electrophoresis.** The proteins in the extract were separated on 7% polyacrylamide gels at pH 8.3, according to the method used by Davis (1964). A volume of 100  $\mu$ l, corresponding to 200–400  $\mu$ g protein, was applied to the top of the running gel.

The electrode buffer was mixed with bromphenol blue for detection of the front after staining. The electrophoresis was conducted at 2 mA per tube for 90 minutes. The enzymes were detected by incubation in solutions containing a specific substrate and stain for each enzyme. For all the reactions, test solutions without substrate or cofactor served as controls. The GDH activity was determined in the direction of  $\alpha$ -ketoglutarate production using  $NAD^+$  as a co-enzyme (Fine and Costello 1963). GOT was visualized by using aspartate and  $\alpha$ -ketoglutarate as substrates and fast violet B as dye (Brewbaker *et al.* 1968). No method on gels was found for GPT. After being stained, the gels were photographed. Densitometer tracings of the gels were obtained by use of a lin/log densitometer (Kipp & Zonen DD691-E). A red filter with maximum transmittance at 580–650 nm was used for scanning the enzyme patterns of GDH. A blue filter with maximum transmittance at 375–425 nm was used for GOT. Quantitative calculations from densitometer curves were carried out by measuring the curve area. The densitometer curve area was directly proportional to the amount of sample applied to the gel within the range between 50 and 150  $\mu$ l protein extract corresponding to 50–400  $\mu$ g protein (Figure 1).

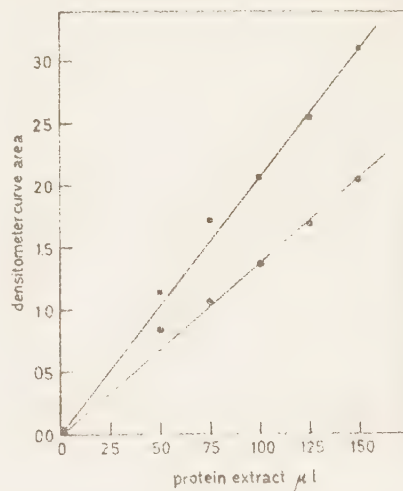


Figure 1. The densitometer curve area (relative units) as a function of the amount of protein applied to the gel. (■) Aspartate aminotransferase, (●) glutamate dehydrogenase ( $NAD^+$ -dependent).





**Spectrophotometric measurements.** A Beckman DK2 spectrophotometer was used for all reactions described below. The activity of GDH was determined in the direction of glutamate formation using NADH as a coenzyme (Schmidt 1963). Instead of triethanolamine buffer (with EDTA), Tris-HCl buffer was used, and EDTA was excluded, since this substance causes a decrease in the enzymatic activity (Lefranc, Welander and Vieira da Silva 1972). Calculations of the GDH activity were made according to Bücher (Schmidt 1963).

The GOT activity was measured with aspartate and  $\alpha$ -ketoglutarate as substrates. Keto acid production was defined by formation of dinitrophenylhydrazones and measurement of absorbance at 546 nm (Bergmeyer and Bernt 1962). GTP was determined in the same way as GOT, but alanine was used as substrate instead of aspartate.

For soluble protein determination, the method employed by Potty (1969) was used after TCA-precipitation of the proteins. Kjeldahl analysis of the total nitrogen could not be used because PVP (included in the grinding buffer) is nitrogenous. Phenols give a positive reaction with the Lowry method (1951) so that the method was not applicable, since most plant material contains large amounts of phenols.

The non-protein  $\alpha$ -amino nitrogen was determined colorimetrically according to Moore and Stein, as described by Lexander *et al.* (1970). The total protein nitrogen of the dry matter was determined according to Kjeldahl after TCA precipitation.

Results

Figure 2 indicates that the amount of protein per gram dry weight was larger in young leaves than in old ones, whereas the amount of non-protein  $\alpha$ -amino nitrogen increased with leaf age. The amount of protein per gram dry weight and non-protein  $\alpha$ -amino nitrogen seemed to be rather insensitive to both daylength and incident light energy. Small changes, however, could be detected with increasing total light energy.

GDH separated on polyacrylamide electrophoresis and visualized as described in Materials and Methods showed several bands on the gels (Figure 3). In the absence of substrate, sodium glutamate, no bands were visible. When the cofactor NAD<sup>+</sup>, necessary for GDH activity, was omitted, two bands were still visible. These two bands represent other enzymes able to oxidize glutamic acid. Such NAD<sup>+</sup>-

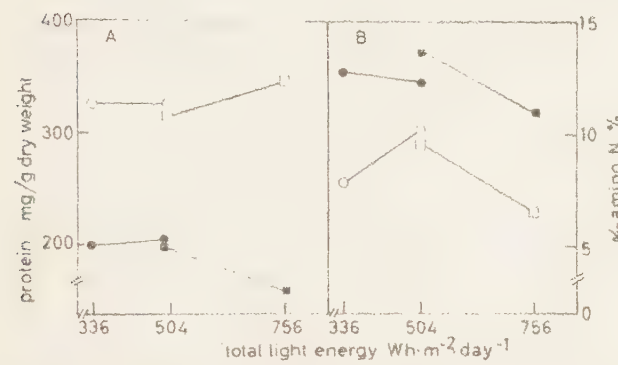


Figure 2. The effect of daylength and total light energy on (A) the amount of protein per gram dry weight, (B) the content of non-protein  $\alpha$ -amino nitrogen expressed as percentage of total nitrogen. Each point is a mean of three replicates. The values correspond to cultivation II. (○) short day, young leaves; (□) long day, young leaves; (●) short day, old leaves; (■) long day, old leaves.

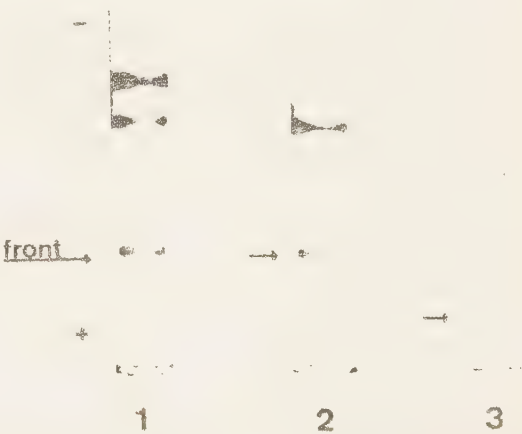
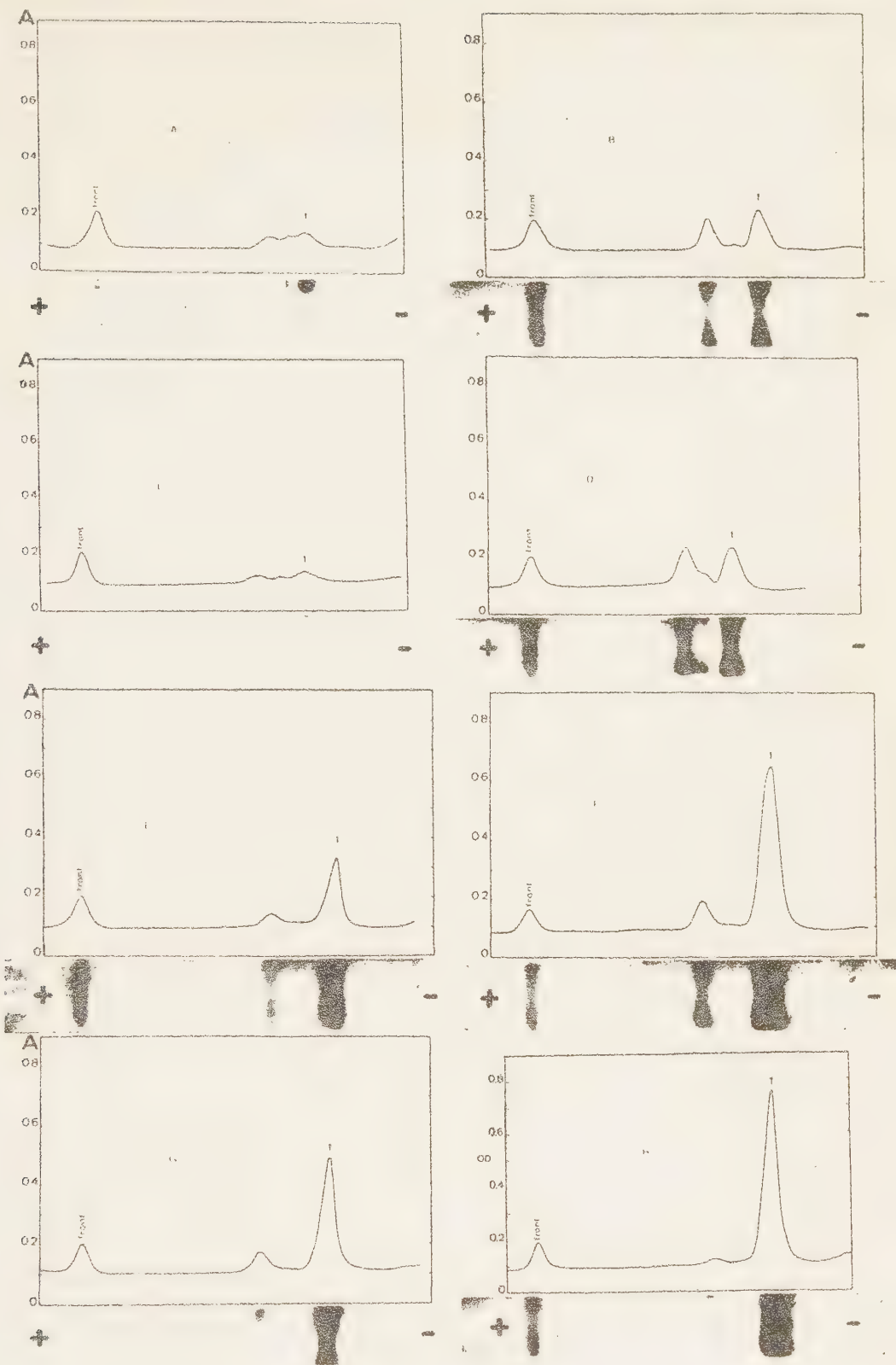


Figure 3. Glutamate dehydrogenase (NAD<sup>+</sup>-dependent) separated by polyacrylamide gel electrophoresis. (1) Total reaction medium, (2) without NAD<sup>+</sup>, (3) without glutamic acid.

Figure 4 (next page). Photographs and densitometric traces of glutamate dehydrogenase (NAD<sup>+</sup>-dependent) after polyacrylamide gel electrophoresis. The gels received 100  $\mu$ l protein extract each. The values correspond to cultivation I.

| Part-figures | Daylength and irradiance                | Leaf age | $\mu$ g protein/100 $\mu$ l extract. |
|--------------|-----------------------------------------|----------|--------------------------------------|
| 4A           | short day 28 W $\times$ m <sup>-2</sup> | young    | 285                                  |
| 4B           | long day 28 W $\times$ m <sup>-2</sup>  | young    | 123                                  |
| 4C           | short day 42 W $\times$ m <sup>-2</sup> | young    | 277                                  |
| 4D           | long day 42 W $\times$ m <sup>-2</sup>  | young    | 155                                  |
| 4E           | short day 28 W $\times$ m <sup>-2</sup> | old      | 176                                  |
| 4F           | long day 28 W $\times$ m <sup>-2</sup>  | old      | 90                                   |
| 4G           | short day 42 W $\times$ m <sup>-2</sup> | old      | 198                                  |
| 4H           | long day 42 W $\times$ m <sup>-2</sup>  | old      | 115                                  |







independent enzymes have been described by Tsukamoto (1962). The number and activity of NAD<sup>+</sup>-independent enzymes varied with leaf age and alteration of growth conditions (Figure 4). Variations with leaf age was earlier shown by Lefranc, Welander and Vieira da Silva (1972). These observations show that extreme caution must be taken when GDH bands are interpreted. Consequently, it is important to note that multiple electrophoretic zones need not necessarily indicate isoenzymes.

All the densitometer curves concerning GDH (Figure 4) showed one uniform NAD<sup>+</sup>-dependent enzyme indicated by the number 1. The other peaks are characterized by independency of the coenzyme NAD<sup>+</sup>. Plants exposed to short days (Figure 4A, C, E, G) showed much lower GDH activity than did those subjected to long days (Figure 4B, D, F, H). The highest GDH activity was obtained in old leaves during long days (Figure 4F, H). In long days high GDH activity was observed in both young and old leaves. Leaves exposed to short days and the irradiance of 42 W × m<sup>-2</sup> (Figure 4C, G), received the same total light energy as leaves exposed to long days and the irradiance of 28 W × m<sup>-2</sup> (Figure 4B, F). Thus, the variation in the GDH activity obtained under the above-mentioned conditions could not be caused only by differences in the incident light energy.

The GDH activity increased with leaf age as presented in Table 1, where five pairs of leaves were investigated with polyacrylamide gel electrophoresis. The daylength treatments caused differences in enzyme activity (Figures 4, 5) as well as differences in leaf number and in the formation of floral shoots. For this reason, the batch of plants used in Table 1 was kept 3 weeks extra under short-day conditions (42 W × m<sup>-2</sup>), at which time they corresponded to the long-day (28 W × m<sup>-2</sup>) plants in their leaf number. Young leaves from plants exposed to short days showed a GDH activity similar to that of young leaves from plants harvested 3 weeks later under the same conditions. Thus, these data also indicate that differences in the enzymatic activity, obtained in young leaves under long- and short-day conditions, were not caused by differences in leaf number.

Table 1. Variations in glutamate dehydrogenase activity obtained from leaves of different ages. The plants were kept under short-day conditions (42 W × m<sup>-2</sup>) for 3 weeks longer than the main harvest. Mean value of three tests.

| Leaf number counted from the bottom | GDH activity, relative units<br>Densitometer curve area/mg protein |
|-------------------------------------|--------------------------------------------------------------------|
| First                               | 4.26                                                               |
| Third                               | 4.33                                                               |
| Fifth                               | 1.16                                                               |
| Seventh                             | 1.44                                                               |
| Eighth (expanding)                  | 0.15                                                               |

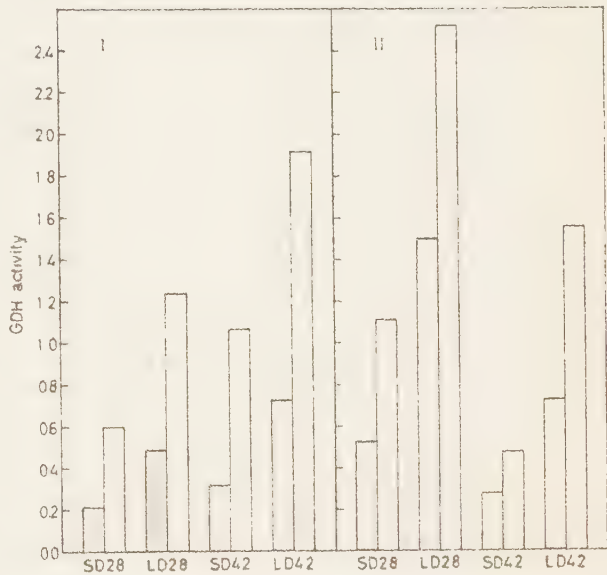


Figure 5. Spectrophotometric measurements of glutamate dehydrogenase (NADH-dependent) activity from young and old leaves under different growth conditions. Dotted bars: young leaves; white bars: old leaves. SD: short day; LD: long day; 28 and 42: irradiance in W × m<sup>-2</sup>. Ordinate: GDH activity in units per mg protein. (I) Cultivation I; (II) Cultivation II.

Table 2. The distribution of NADH- and NAD<sup>+</sup>-dependent GDH activity in young and old leaves as percentage of total activity under different growth conditions. SD: short day; LD: long day; 28 and 42: irradiance in W × m<sup>-2</sup>.

| Growth conditions | Leaf age | Spectrophotometric measurements<br>NADH-dep. GDH<br>% | Electrophoretic measurements<br>NAD <sup>+</sup> -dep. GDH<br>% |
|-------------------|----------|-------------------------------------------------------|-----------------------------------------------------------------|
| SD 28             | young    | 28.5                                                  | 13.6                                                            |
| SD 28             | old      | 71.5                                                  | 86.4                                                            |
| LD 28             | young    | 27.5                                                  | 13.9                                                            |
| LD 28             | old      | 72.5                                                  | 86.1                                                            |
| SD 42             | young    | 26.7                                                  | 14.0                                                            |
| SD 42             | old      | 73.9                                                  | 86.0                                                            |
| LD 42             | young    | 23.3                                                  | 8.4                                                             |
| LD 42             | old      | 76.7                                                  | 91.6                                                            |





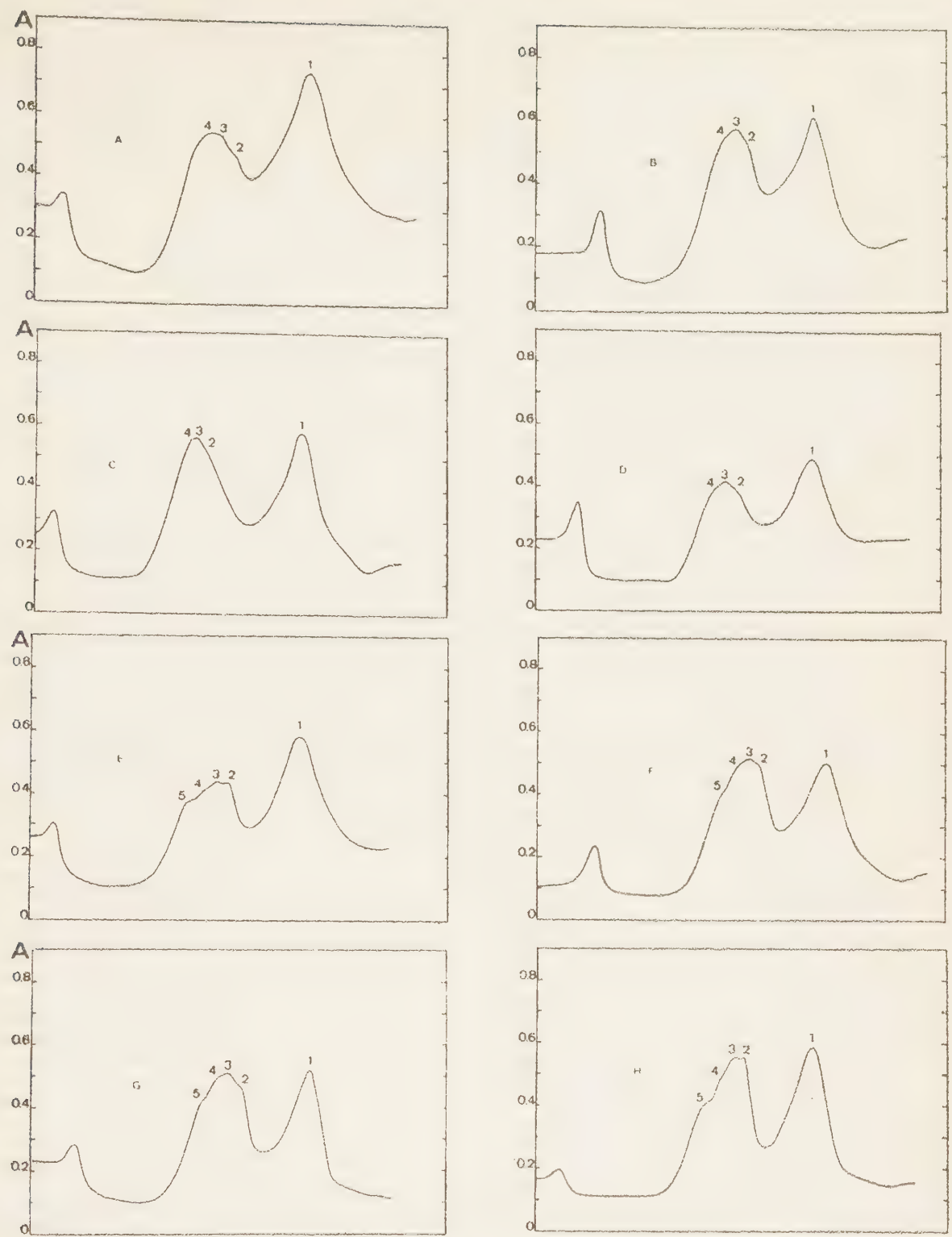


Figure 6. Densitometer traces of aspartate aminotransferase after polyacrylamide gel electrophoresis. The gels have received 100  $\mu$ l protein extract each. Arrangement of part-figures as in Figure 4. The values correspond to cultivation I.



Figure 5 represents the GDH activity recorded as spectrophotometric measurements from two cultivations (I and II). The densitometer curves (Figure 4) correspond to cultivation I. The GDH activity was highest under long-day conditions and increased with leaf age.

The values from Figure 5 indicate that the ratio between the GDH activity of old and that of young leaves remained rather constant both within and between the two cultiva-

tions independently of the growth conditions ( $2.7 \pm 0.3$  within cultivation I and  $1.9 \pm 0.2$  within cultivation II). This was also the case for the ratios between the GDH activity of leaves of the same age taken from different plants subjected to either short or long days and one of two different irradiances ( $2.1 \pm 0.2$  within cultivation I and  $2.8 \pm 0.4$  within cultivation II).

Table 2 indicates that the distribution of NADH- and NAD<sup>+</sup>-dependent GDH activity between young and old leaves was similar under different growth conditions.

The densitometer curves from electrophoretic separations of GOT (Figure 6) indicate no outstanding quantitative differences, neither between young and old leaves nor between leaves exposed to short or long days and different light energies. Slight variations in the curves probably depend on different amounts of protein in the samples. Five isoenzymes could be detected in old leaves (Figure 6E, F, G, H) but only four in young leaves (Figure 6A, B, C, D). The results from the spectrophotometer confirmed that the GOT activity remained rather constant throughout the whole experiment although the activity tended to rise in old leaves.

The GPT activity (Figure 7) increased during long days in the same way as GDH. Figure 8 shows the relationship between GDH and GPT activity in young and old leaves from two cultivations (I and II). It is evident that a correlation exists between the enhancement of GDH activity and that of GPT in *Urtica dioica*.

## Discussion

Plants under long-day conditions show much higher GDH activity than those kept during short days. Plants exposed to long days are flowering. The activity of GDH also increases with leaf age. This indicates that long days, or the flowering induced by long days, creates conditions similar to those appearing in old leaves. The results from spectrophotometric measurements of GPT indicate that this enzyme responds to photoperiodic induction in the same way as GDH. The GPT activity is highest in plants exposed to long days and increases with leaf age. A high correlation between GDH and GPT activity was established with a correlation coefficient of 0.928. GDH and GPT produce either  $\alpha$ -ketoglutarate and/or glutamate which could act as substrates for the two enzymes and stimulate the enzyme activities.

Hedley and Stoddart (1971) demonstrated that GPT activity increases to a maximum value during both short days and inductive long days. Under short-day conditions this activity remains constant, but during long days the activity declines after floral induction. In later investigations Hedley and Stoddart (1972) stated that even during short days the GPT activity declines but later than during long days. Plants exposed to short days never reach as high GPT activity as plants subjected to long days.

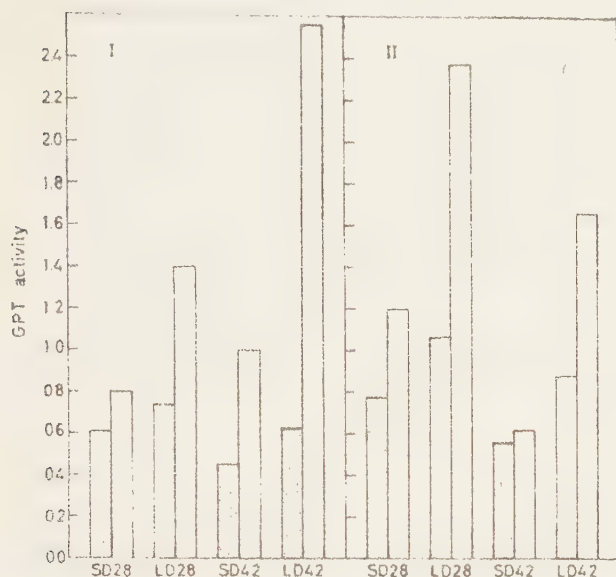
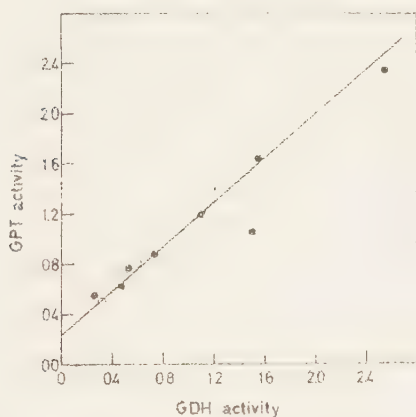


Figure 7. Spectrophotometric measurements of alanine aminotransferase activity from young and old leaves under different growth conditions. Abbreviations as in Figure 5. Ordinate: GPT activity in  $\mu\text{mol}$  keto acid produced per h and mg protein. (I) Cultivation I; (II) Cultivation II.





There is a continuous "turnover" of proteins in leaves (Steward and Durzan 1965). During the ageing of the leaves there is a sequence in the build-up of the individual amides and amino acids in the soluble phase of the leaf (Pate 1966). Well-defined rhythms in the enzyme activity and in the amounts of specific metabolites create the impression that ageing may involve progressive alteration in the balance of anabolic and catabolic compounds. Figure 2 indicates that accumulation of free amino acids increases with leaf age. In experiments with *Xanthium pennsylvanicum*, Krizek, McIlrath and Vergara (1966) demonstrated that the kind of leaf senescence that follows a photoperiodically induced transition from vegetative to reproductive conditions occurs whether or not the plants had a capacity to produce flowers or fruits. This type of leaf senescence seems to result from photoperiodic effects on the leaves themselves.

The GOT activity does not respond to environmental changes. This seems to reflect a rather general state of the GOT activity. Hedley and Stoddart (1971) demonstrated that *Lolium temulentum*, exposed to inductive photoperiods, maintains a constant GOT activity.

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## The Effect of Mercaptoethanol on the Activity of Enzymes of Nitrogen Metabolism in Leaves from *Urtica dioica* and *Spinacia oleracea*

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### Abstract

The effect of mercaptoethanol at different concentrations on enzyme activity was investigated in leaves from *Urtica dioica* and *Spinacia oleracea*. The interference of mercaptoethanol with enzyme activity is dependent on the type of plant, the configuration of the enzyme and the concentration of mercaptoethanol. A stimulation of GDH (glutamate-dehydrogenase) was obtained in both species, while inhibition of GOT (glutamate-oxaloacetate transaminase) and GPT (glutamate-pyruvate transaminase) was demonstrated in *Spinacia*. The stimulation of GOT and GPT activity in *Urtica* was probably due to inhibition of phenol oxidase. Conclusions concerning the effect of mercaptoethanol on NR (nitrate reductase) activity were difficult to draw, since mercaptoethanol itself reduced nitrite and interfered with NR determination in tests *in vitro*. In *Urtica*, no activity could be obtained at all with the *in vitro* method, probably because an inhibitor of NR was liberated during the extraction procedure. Activity of NR could however be obtained in both species when using the *in vivo* method. Addition of protective agents to the extraction medium has been supposed to influence the protein extractability. In conformity with this increasing amount of fresh matter to the same volume of extraction medium resulted in decreased protein extractability. This led to differences in enzyme activity when expressed on a fresh weight basis but the specific activity remained constant.

### Introduction

The presence of phenolic compounds and high activity of phenol oxidases in plant tissues results in browning of extracts and prevents isolation of active enzymes. Loomis and Battaile (1966) reviewed the chemistry of these reactions and the use of insoluble polyvinylpyrrolidone (PVP). PVP adsorbs phenols from plant tissue extracts. Using this compound, they were able to obtain active, soluble enzymes from leaves of peppermint, thistle and apple. To minimize browning and increase protein extractability, many other protective agents have been used besides PVP. Tucker and Fairbrothers (1970) have compared 17 phenol-removing, phenol-oxidase-inhibiting, and quinone-removing reagents in

serial dilutions in order to find out the best method of extracting proteins from leaves of *Mentha*. The best results were obtained with 0.25 M 2-mercaptoethanol. The proteins extracted were measured by disc-electrophoresis, alkaline phosphatase activity and total protein content. Klepper and Hageman (1969) have been able to demonstrate nitrate reductase activity in leaves from apple. Besides PVP, they also used 2-mercaptobenzothiazole, a phenol oxidase inhibitor, in the extraction medium. Dirr *et al.* (1972) could not detect any nitrate reductase activity in extracts from blueberry and other ericaceous plants with a high content of phenols, although PVP had been added during homogenization. When they employed a modified method according to Mulder *et al.* (1959) and using sectioned leaves, nitrate reductase activity could be obtained. In work with *Urtica dioica* (Welander 1974), several enzymes were investigated. Leaf extract from this species turned brown immediately, and no enzyme activity could be obtained without PVP. For assay of glutamate dehydrogenase, mercaptoethanol in addition to PVP, was also required in the extraction medium.

Mercaptoethanol is believed to act as a quinone-removing substance. In higher concentrations, it will react with proteins by disrupting disulfide bridges. For this reason, it seemed important to investigate the effect of mercaptoethanol on the activity of different enzymes. The enzymes investigated were glutamate dehydrogenase (GDH, E.C. 1.4.1.2.), glutamate-oxaloacetate transaminase (GOT, aspartate aminotransferase E.C. 2.6.1.1.), glutamate-pyruvate transaminase (GPT, alanine aminotransferase E.C. 2.6.1.2.), and nitrate reductase (NR, NADH: nitrate oxidoreductase E.C. 1.6.6.1.). Two species, *Urtica dioica* and *Spinacia oleracea*, were compared. Due to difficulties in obtaining NR activity in species with a high content of phenols, two methods were used: *In vitro* (with homogenized leaves) and *in vivo* (with sectioned leaves).

Protective agents against phenols and quinones added to the extraction medium are supposed to increase protein



extractability. How the ratio of the amount of fresh matter to the volume of extraction medium influences protein extractability and the way enzyme activity is expressed, was investigated.

**Abbreviations:** GDH, glutamate dehydrogenase; GOT, glutamate-oxaloacetate transaminase; GPT, glutamate-pyruvate transaminase; NR, nitrate reductase; NADH, nicotinamide adenine dinucleotide reduced; PVP, polyvinylpyrrolidone; TCA, trichloroacetic acid.

### Material and Methods

#### *Plant material and cultivation*

*Urtica dioica* L. (wild, Sweden) and *Spinacia oleracea* L. were grown in 4 litre pots in soil mixed with 10 grams of Weibull's Enpekå special fertilizer (12% N, 12% P<sub>2</sub>O<sub>5</sub>, 19% K<sub>2</sub>O and micro-nutrients). Each pot contained 10 plants of either *Urtica* or *Spinacia*. The plants were raised in climate chambers maintained at 22°C during 18 h photoperiod and at 12°C at night. The plants were subjected to an irradiance of 30 W·m<sup>-2</sup> from cool-white fluorescent lamps (F48PG17/CWC, General Electric). The experiments included 30 plants of each species and the plant age was 50 days at harvest.

#### *Storage of mercaptoethanol*

During the experiments with mercaptoethanol, it turned out to be very important to store mercaptoethanol over nitrogen. When in contact with oxygen, mercaptoethanol is oxidized to dithiodiethanol. Mercaptoethanol has an absorbance maximum at 235 nm in alkaline milieu (Dekan *et al.* 1956), while the disulfide has a shoulder between 230–260 nm at pH 6.5 (Coleman *et al.* 1968). Thus, the oxidation process can be checked.

#### *Sampling and protein extraction*

Mature leaves from each plant were used. In order to obtain samples as uniform as possible, leaves from 30 plants were collected and cut together. For each extraction, samples of 4 g of fresh weight from the cut leaf material were used. The details of the extraction method were as described by Welander (1974). The extraction medium consisted of 0.1 M K-phosphate buffer and mercaptoethanol at seven different concentrations in the interval 0–0.85 M. All the protein extracts used for enzyme determinations were purified on Sephadex G25 columns, except extracts for NR assays. The enzyme determinations were carried out immediately after protein extraction. Two parallel extractions were performed at the same time in each experiment.

#### *Enzyme determinations*

A Varian Techtron UV-VIS spectrophotometer model 635 was used for all enzyme assays. The activities of GDH, GOT

and GPT were measured as described by Welander (1974), except that Tris-HCl buffer was replaced by K-phosphate buffer. The activity of GDH was expressed in units calculated according to Bücher (Schmidt 1963). The activities of GOT and GPT were expressed in units defined as  $\mu\text{mol}$  ketoacid produced per h. The activity of NR was measured by both the *in vitro* method according to Hageman and Hucklesby (1971) and the *in vivo* method according to Jaworski (1971). In the *in vitro* method, the test mixture was modified to contain 0.1 M K-phosphate buffer (25  $\mu\text{mol}$ ), 0.1 M KNO<sub>3</sub> (25  $\mu\text{mol}$ ), 2 mM NADH (0.5  $\mu\text{mol}$ ) and 0.5 ml enzyme solution. The reaction was initiated by the addition of the enzyme extract. A test solution without NADH was used as a control. After incubation at 30°C for 20 min, the reaction was terminated by the addition of 0.1 ml of 1 M zinc acetate and 0.9 ml of cold ethanol (70%). The test tubes were vigorously shaken and maintained at 0°C for 10 min. Precipitation with zinc acetate removes the excess of NADH which may later interfere with colour formation. After centrifugation at 3000 g for 10 min, 2 ml of the supernatant was used for assay of nitrite by the addition of 1 ml sulfanilamide (1% in 3 M HCl) and 1 ml N-(naphthyl) ethylenediamine hydrochloride (0.02%). The red colour formed was allowed to develop for 30 min and the absorbance was measured at 540 nm. The activity was expressed as  $\mu\text{mol}$  NO<sub>2</sub><sup>-</sup> produced per mg of protein and h. In the *in vivo* method, the activity was expressed as  $\mu\text{mol}$  nitrite produced per g fresh weight and h. All enzyme assays were repeated three times.

#### *Protein determination*

All protein assays were measured as described by Potty (1969) after TCA-precipitation of the proteins. The following assay was made to test how the use of different amounts of fresh matter to the same volume of extraction medium influences protein extractability and the way enzyme activity is expressed. Four different amounts of fresh matter (0.5, 1, 2, 2.5 g) of leaves from *Urtica* were extracted with 10 ml extraction medium as described above in "Sampling and protein extraction". The activity was expressed on either fresh weight basis or on protein basis.

### Results

The results show that mercaptoethanol in different concentrations interferes with the activity of the investigated enzymes. GDH activity in leaves from both *Urtica* and *Spinacia* was stimulated by mercaptoethanol to a certain level (Figure 1). For *Spinacia* maximum activity was reached at a concentration of 0.14 M mercaptoethanol and remained constant to 0.56 M, whereupon the activity decreased. GDH in *Urtica* attained a maximum at a concentration of 0.28 M mercaptoethanol, after which the activity remained at a constant level. Isolation of GDH from *Urtica* obviously





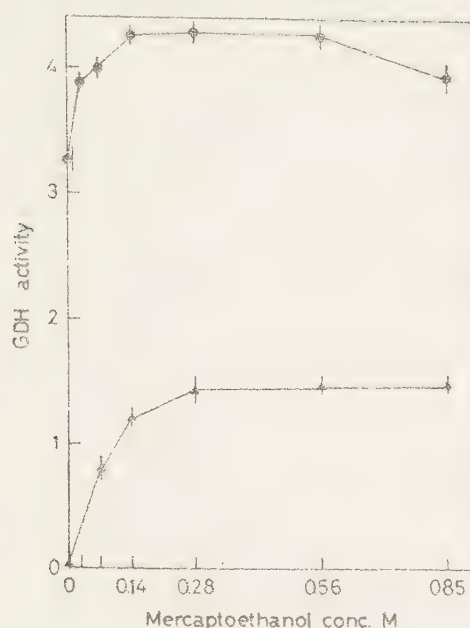


Figure 1. The effect of mercaptoethanol in the extraction medium on glutamate dehydrogenase activity. (●) *Spinacia oleracea*, (▲) *Urtica dioica*. Purified extracts were used for GDH determinations. Ordinate: GDH activity in units per mg protein. Each plot represents a mean value. The vertical bars denote the differences between data obtained from two parallel extractions with three analyses for each.

requires mercaptoethanol in the extraction medium to preserve activity of the enzyme.

Mercaptoethanol inhibited GOT activity in leaf extract from *Spinacia* at all the investigated concentrations (Figure 2). The activity of GOT in *Urtica*, on the contrary, was highest at a concentration of 0.07 M mercaptoethanol.

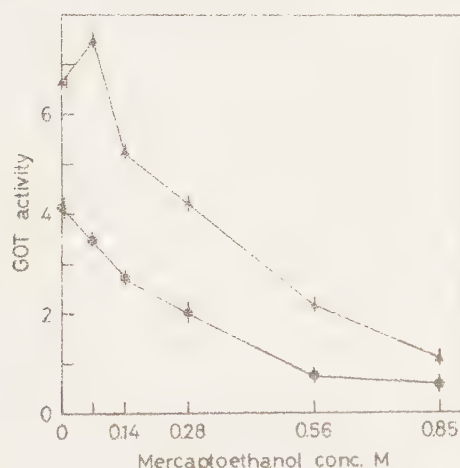


Figure 2. The effect of mercaptoethanol in the extraction medium on glutamate-oxaloacetate transaminase activity. (●) *Spinacia oleracea*, (▲) *Urtica dioica*. Purified extracts were used for GOT determinations. Ordinate: GOT activity in units per mg protein. Data expressed as in Figure 1.

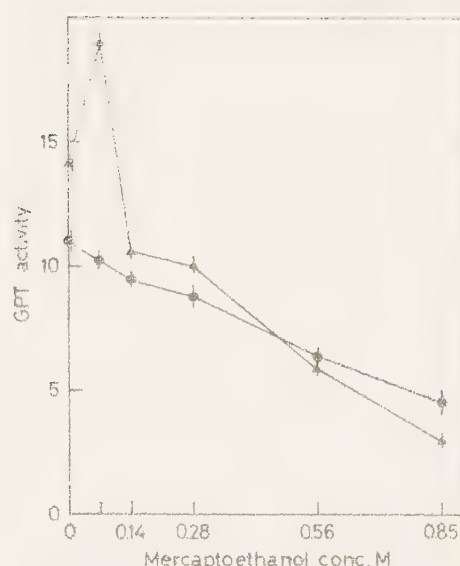


Figure 3. The effect of mercaptoethanol in the extraction medium on glutamate-pyruvate transaminase activity. (●) *Spinacia oleracea*, (▲) *Urtica dioica*. Purified extracts were used for GPT determinations. Ordinate: GPT activity in units per mg protein. Data expressed as in Figure 1.

Higher concentrations of mercaptoethanol lead to a decrease in the activity of GOT in *Urtica*.

Figure 3 illustrates the influence of mercaptoethanol on GPT activity in *Spinacia* and *Urtica*. The results were similar



Figure 4. The effect of mercaptoethanol in the extraction medium on nitrate reductase activity. Crude extracts were used for NR determinations. (●) *Spinacia oleracea*, (▲) *Urtica dioica*. Ordinate: NR activity in  $\mu\text{mol}$  nitrite produced per h and mg protein. Each plot represents a mean value of three analyses.



Table 1. Nitrate reductase activity.

| Plant species            | <i>In vitro</i> method<br>$\mu\text{mol NO}_2^-$ per mg<br>protein and h<br>(mean value of<br>three analyses) | <i>In vivo</i> method<br>$\mu\text{mol NO}_2^-$ per g<br>fresh weight and h<br>(mean value $\pm$ SD,<br>n = 5) |
|--------------------------|---------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------|
| <i>Urtica dioica</i>     | 0                                                                                                             | $1.33 \pm 0.04$                                                                                                |
| <i>Spinacia oleracea</i> | 1.20                                                                                                          | $0.54 \pm 0.01$                                                                                                |

to those obtained for GOT. The activity of GPT in *Urtica* was highest at a concentration of 0.07 M mercaptoethanol and then decreased. GPT activity in *Spinacia* decreased with increasing mercaptoethanol concentration.

The effect of mercaptoethanol on NR activity is shown in Figure 4. In *Spinacia* the apparent activity of NR diminished substantially at any investigated concentration of mercaptoethanol. No NR activity could be detected at all in *Urtica* at any concentration of mercaptoethanol. The harmful effect of mercaptoethanol on apparent NR activity in *Spinacia* is probably not due to inhibition of the enzyme itself. In the *in vitro* method, the amount of nitrite produced is a measure of NR activity. Mercaptoethanol is a strong reducing agent and its ability to reduce nitrite was established. In an assay where different concentrations of mercaptoethanol were added to a known amount of nitrite (0.35 mM), it was shown that a concentration of 0.028 M mercaptoethanol reduces the amount of nitrite by about 80%. The inability to detect the

activity of NR in *Urtica* is probably due to an endogenous inhibitor liberated during the extraction procedure. Activity of NR could be obtained by the *in vivo* method for both *Urtica* and *Spinacia* (Table 1).

Figure 5 shows that the extractability of proteins decreased when increasing amounts of fresh matter from *Urtica* were used to the same volume of extraction medium. This results in differences in enzyme activity of GOT and GDH if the activity is expressed on a fresh weight basis. The specific activity remains constant.

### Discussion

Browning of plant tissue extracts, caused by phenolic compounds and their oxidation products, prevents isolation of active enzymes (Loomis and Battaile 1966, Tucker and Fairbrothers 1970). Varying amounts of phenols and different activity of phenol oxidase have been demonstrated by Esen and Soost (1974) in non-browning and browning taxa in *Citrus*. Non-browning taxa lacked the substrate and had no or little phenol oxidase activity. Leaf extracts from *Urtica* turn brown immediately after extraction if no protective agents are added. Leaf extracts from *Spinacia* remain green, which indicates low activity of phenol oxidase and/or low content of phenols. Figure 1 shows that GDH activity was stimulated by mercaptoethanol in leaf extracts from both *Urtica* and *Spinacia*. In addition, isolation of GDH from *Urtica* requires mercaptoethanol in the extraction medium to obtain activity of the enzyme. It is suggested that mercaptoethanol included in extract from *Urtica* has a dual effect. On the one hand, mercaptoethanol seems to inhibit phenol oxidase and on the other hand stimulates the activity of GDH. This may explain the different effects of mercaptoethanol on GDH from *Spinacia* and from *Urtica*.

Activation of GOT and GPT occurred only in leaf extracts from *Urtica*, and for both enzymes at the same concentration of mercaptoethanol (Figures 2 and 3). Thus the increased activities of GOT and GPT are probably due to inhibition of phenol oxidase and not to stimulation of the enzymes themselves.

In *Urtica*, the stimulative effect of 0.07 M mercaptoethanol is higher on the activity of GPT than for GOT. In *Spinacia*, the inactivation of GOT and GPT activity with increasing concentration of mercaptoethanol is more pronounced for GOT than for GPT. Thus GOT and GPT differ in sensitivity to mercaptoethanol. Among possible explanations are different sensitivity to reagents that modify SH-groups in proteins and to different sensitivity to changed redox potential in the extraction medium. Loomis and Battaile (1966) reported the effect of 0.25 M mercaptoethanol on enzyme activity in leaf extracts from peppermint. Very low activity of mevalonic kinase was obtained with mercaptoethanol in the extraction medium. No differences in enzyme activity were found between crude extract and

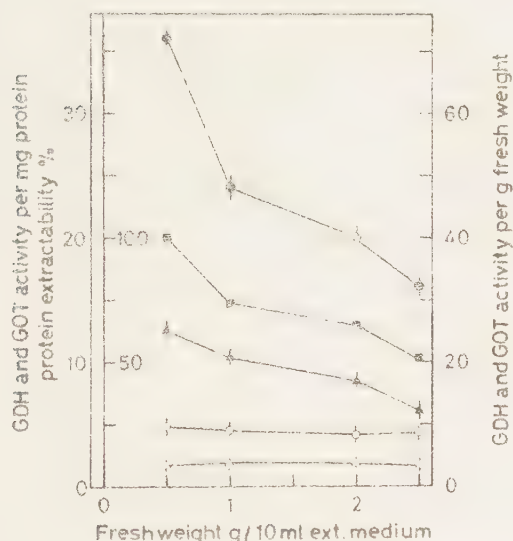


Figure 5. The influence of increasing amounts of fresh matter per 10 ml extraction medium on protein extractability and on the way enzyme activity is expressed. Ordinates: (○) glutamate-oxaloacetate transaminase and (△) glutamate dehydrogenase activity expressed as units per mg protein, (●) glutamate-oxaloacetate transaminase and (▲) glutamate dehydrogenase activity expressed as units per g fresh weight. (■) protein extractability; the amount of protein extracted from sample of 0.5 g fresh weight represents 100%. Data expressed as in Figure 1.



extract purified by gel filtration. This indicates that the harmful effect of mercaptoethanol is due to changes in protein configuration which could not be reversed by gel filtration. On the other hand, high activity of alkaline phosphatase could be obtained with mercaptoethanol in the extraction medium after gel filtration of crude extract. The effect of mercaptoethanol in this case could be due to changed redox potential in the test medium which could probably be reversed by gel filtration. The results of Loomis and Battaile (1966) and those given here for GDH, GOT and GPT may thus indicate that interference of mercaptoethanol with enzyme activity is dependent on both protein configuration — probably due to disruption of disulfide bridges of the proteins — and on redox effects of mercaptoethanol.

It was difficult to draw any conclusions concerning the effect of mercaptoethanol on the activity of NR. This because mercaptoethanol itself reduced nitrite, used as a measure on NR activity in tests *in vitro*. In *Urtica* no activity at all could be obtained with the *in vitro* method (Figure 4), probably because an inhibitor of NR was liberated during the extraction procedure. Endogenous inhibitors of NR have previously been reported. Stulen *et al.* (1971) demonstrated an inhibitor, probably of phenolic nature, with respect to NR in radish cotyledons. Dirr *et al.* (1972) could not detect NR in homogenized leaves from two ericaceous species, although they had reduced the phenol content with PVP. When they employed a modified *in vivo* method according to Mulder *et al.* (1959), NR activity could be detected. Dirr *et al.* (1972) suggested that other compounds besides phenols could inhibit NR.

Protective agents added to the extraction medium has been supposed to change protein extractability in plant material rich in phenols and having high activity of phenol oxidase (Tucker and Fairbrothers 1970). The results given in Figure 5 show that changed protein extractability might lead to errors if the enzyme activity is expressed on a fresh weight basis but not on a protein basis.

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The influence of photoperiodic variation  
on plant morphogenesis and nitrogen metabolism  
in *Urtica dioica* and *Spinacia oleracea*

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## Introduction

The main purpose of the present study is to investigate the effect of photoperiodic variation on plant morphogenesis and protein production, and possible correlations between protein production and enzymes involved in nitrogen metabolism. As shown earlier by Welander (1974), the activity of GDH and GPT in leaves from *Urtica dioica* is affected by the photoperiodic length. Leaf extracts from *Urtica* immediately turn brown if no protective agents are added during the extraction procedure. Mercaptoethanol was found to be the most efficient agent in order to inhibit browning. On the other hand, this substance also interferes with the activity of several enzymes (Welander 1978). In the present investigation, therefore, *Urtica dioica* is compared with *Spinacia oleracea*, the leaf extracts of which did not display any browning. The enzymes here studied are glutamate dehydrogenase (GDH, E.C. 1.4.1.2.), glutamate-oxaloacetate transaminase (GOT, aspartate aminotransferase E.C. 2.6.1.1.), glutamate-pyruvate transaminase (GPT, alanine aminotransferase E.C. 2.6.1.2.), and nitrate reductase (NR, NADH: nitrate oxidoreductase E.C. 1.6.6.1.).

The growth habit of plants exposed to either long or short photoperiods is often profoundly altered (Vince Prue 1975). The photoperiodic effect on the vegetative growth of some vegetable crops was studied by Milford and Lenton (1976) and Soffe et al. (1977). An extension of the photoperiod with photosynthetically useful light was found to increase dry weight and leaf area in proportion to total dry matter. There was no change in the way the incremental dry matter was distributed within the plant. An extension of the daylength with photosynthetically active light has been found to affect the rate of photosynthesis, thus altering the rate of formation of carbon skeletons available for amino acid biosynthesis. The close relationship between photosynthetic products and nitrogen metabolism was established by Steer (1973).





It has been suggested that in higher plants, nitrogen metabolism and nitrate reduction capacity are correlated with protein production (Beevers and Hageman 1969). Nitrate absorbed by plant roots must be reduced before it can combine with keto acids in order to produce amino acids and finally form proteins. Nitrate reductase is the first enzyme and the rate limiting step in the assimilation pathway of nitrate. It is well known that light, temperature, mineral nutrition, plant age as well as genetic composition influence the level of nitrate reductase (Beevers and Hageman 1969, Nicholas et al. 1976). Furthermore, this enzyme is substrate inducible and has a high turnover rate (Zielke and Filner 1971).

Nitrate can be assimilated in both the root and the shoot. The distribution of nitrate assimilation within the plant varies from one species to another. One extreme is *Xanthium pennsylvanicum* which substantially lacks or exhibits very low activity in the roots. The opposite extreme is represented by *Pisum arvense* which reduces almost all nitrate in the roots (Wallace and Pate 1965 and 1967). Recent aspects on the distribution of nitrate reductase activity between roots and leaves are given by Radin (1975, 1977 and 1978). The induction of NR activity in cotton roots was found to be regulated by ammonium ions and certain amino acids, whereas no such regulation was observed in the shoots. The activity of NR in cotton shoots was stimulated by a rise in the nitrate level of the growth medium, whereas in the roots the activity of NR was insignificantly affected. A comparison between soybean (representing the *Pisum* extreme) and cotton (representing the *Xanthium* extreme) showed that, in soybean, there exists some sort of restriction in nitrate flow from roots to shoots, giving rise to a high internal nitrate concentration in the roots, which in turn increases NR activity. In cotton, however, no such restriction was observed in nitrate flow from roots to shoots, giving similar nitrate concentrations in the root and the shoot. Even though the roots in the *Xanthium* type take no major part in the NR activity, the relative abundance of NR in roots and leaves should nevertheless be noted.



GDH has until recently been regarded as one of the most important enzymes for ammonium assimilation. The function of GDH in ammonium assimilation is however now thought to be rather limited because of its low affinity to ammonium, i.e. it has a very high  $K_m(\text{NH}_4^+)$  value. It is assumed that an alternative route for ammonium assimilation occurs at normal intracellular levels, (Mifflin and Lea 1976).

The activity of GDH increases in plants supplied with exogenous ammonia, and may involve the synthesis of different isoenzymes. Some isoenzymes can be related to the anabolic and catabolic functions of GDH (Stewart and Rhodes 1977). Davies and Teixeira (1975) have proposed that the balance between aminating/denitrifying activity of GDH is regulated by the NAD/NADH ratio. Givan (1978) has suggested that the most important role of GDH is to dispose of high ammonia levels supplied exogenously or generated endogenously during protein degradation.

It is well known that transamination is the final step in the biosynthesis of many amino acids. Hedley and Stoddart (1972 a and b) have established a correlation between protein synthesis and changes in the activity of GOT and GPT during seed and leaf development in *Lolium*. They suggested that these enzymes may have an important role in the regulation of protein synthesis during organ development. The activity of GPT in expanding leaves of *Lolium* was found to be sensitive to photoperiodic variations, and the same was demonstrated in leaves from *Urtica* (Welander 1974).



## Materials and methods

### Plant material and cultivation

*Urtica dioica* L. (wild Sweden) and *Spinacia oleracea* L. (cv Dominant Weibull) were used as plant material. Seeds from *Spinacia* were germinated in silica sand (Silver Sand 55) in 0.5 litre pots. One seedling was cultivated in each pot. Since similarly treated seeds from *Urtica* did not germinate, those seeds were instead germinated in soil until the length of the seedlings was about 0.5 cm. The seedlings were then transferred to silica sand. Both *Spinacia* and *Urtica* were cultivated in climate chambers. The *Urtica* plants were exposed to either 18-h (LD) or 12-h (SD) photoperiods. Day temperature was 22°C and night temperature 12°C. Since the *Urtica* seedlings proved to be very sensitive when transferred to silica sand, the low night temperature in the climate chamber was not adopted during the first week. The *Spinacia* plants were subjected to either 16-h (LD) or 8-h (SD) photoperiods, the temperature being permanently maintained at 22°C. The plants were subjected to an irradiance of 28 W·m<sup>-2</sup> from cool-white fluorescent lamps (F48 PG17/CWC, General Electric). The cultivations were performed twice.

The experiments comprised 120 plants of each species. The plant age at harvest was for *Spinacia* 45 days in LD and 56 days in SD and, for *Urtica*, 38 days in LD and 49 days in SD. The seedlings from both *Urtica* and *Spinacia* were watered twice a day with 10 ml of nutrient solution of the following composition: concentrations in mM: MgSO<sub>4</sub>, 2; Ca(NO<sub>3</sub>), 2.5; CaCl<sub>2</sub>, 2.5; K<sub>2</sub>SO<sub>4</sub>, 2.5; K<sub>2</sub>HPO<sub>4</sub>, 5; KH<sub>2</sub>PO<sub>4</sub>, 5; Fe-EDTA, 0.01, and NH<sub>4</sub>NO<sub>3</sub>, 2.5. Trace elements: concentrations in µM: MnCl<sub>2</sub>, 9.1; H<sub>3</sub>BO<sub>3</sub>, 46; CuSO<sub>4</sub>, 3.2; ZnSO<sub>4</sub>, 7.7, and Na<sub>2</sub>MoO<sub>4</sub>, 0.4. The pH of the nutrient solution was 6.1. The solution was supplied in concentrations as required by plant growth. During the first growth period, the above-defined nutrient solution was used in dilutions of 1:4, 1:2 and 1:1. At the end of the growth period, normal





strength and twice normal strength of the nutrient solution was used. Spinacia plants received a total of 104 mg nitrogen per plant and Urtica plants 87 mg nitrogen per plant.

#### Morphological parameters, sampling and protein extraction

Thirty plants from each species were used in order to determine certain morphological parameters. Plant height, number of leaves, total laminar area, and root length were measured in both species as well as internode length in Urtica. Dry weight and fresh weight were measured for five fractions of the plant, viz.: (1) young, not fully expanded leaves, (2) the oldest leaves (without deficiency symptoms), (3) the rest of the leaves, here designated middle leaves, (4) stem plus leaf petioles, and (5) roots. In order to avoid diurnal fluctuations in the enzyme level, all samples, both in SD and LD, were taken 45 min after termination of dark period. Leaves from 30 plants were collected and divided into three fractions, as described above. The roots were carefully rinsed and dried between filter paper before use. The leaves from each group as well as roots were cut and after mixing each sample, 4 gram of fresh weight was used for each extraction. The rest of the plant material was freeze-dried and used for nitrogen analyses. The details of the extraction procedure were as described by Welander (1974). The extraction medium consisted of 0.1 M K-phosphate buffer and mercaptoethanol at a concentration of 0.07 M according to Welander (1978).

#### Chlorophyll and enzyme determination

Chlorophyll measurements were performed on leaves from different age-groups of Urtica. The total amount of chlorophyll in 80% acetone was determined by measuring the optical density at 652 nm according to Arnon (1949).

In vitro assay of NR activity was carried out immediately after protein extraction. GDH, GOT, GPT and NR activity was measured and expressed as described by Welander (1974 and 1978).



Determination of proteins, soluble non-protein nitrogen, nitrate  
nitrogen, and ammonium nitrogen

Soluble proteins were assayed as described by Potty (1969) after trichloroacetic acid (TCA) precipitation of the proteins. The total protein nitrogen of the dry matter was determined in samples of 100 mg freeze-dried material extracted with 20 (10 + 5 + 5) ml of 2.5% TCA (w/v). After filtration, the nitrogen content of the remaining residue was determined according to Kjeldahl. The ammonium nitrogen was collected according to Kjeldahl distillation of TCA-soluble fractions. The non-protein nitrogen in the same fraction was determined after heating according to Kjeldahl. The nitrate nitrogen was reduced to ammonium after the addition of 0.5 gram Devarda's alloy and 7.5 ml magnesiumoxide. The ammonium content was estimated as described above.



## Results

### Morphological characteristics

An extension of the photoperiod with photosynthetically useful light results in a profoundly changed growth habit in *Urtica dioica* and *Spinacia oleracea*. Table 1 shows that *Urtica* growing under LD conditions becomes higher and has longer internodes. Plants subjected to SD produce lateral branches, which means that the total number of leaves changes, no significant differences in the total laminar area being however found. Root length was the same for both SD and LD plants. The chlorophyll content in leaves of different age groups in *Urtica* increases with extended photoperiod (Table 2). The highest chlorophyll level was found in old leaves in LD and in middle leaves in SD. *Spinacia* is a rosette plant when growing under SD conditions. LD growth causes elongation of the stem. The total laminar area increases in LD, whereas only small differences in the number of leaves are found between LD and SD. Roots grow longer under LD conditions. Both *Urtica* and *Spinacia* flower when subjected to LD.

The distribution of the amount of fresh weight and dry weight in different plant parts as well as the differences between plants subjected to LD or SD are shown for *Urtica* in Figure 1 and for *Spinacia* in Figure 2.

In *Urtica*, the total amount of fresh weight per plant is larger during LD. On the other hand, the contribution of different plant parts to the amount of fresh weight differs in LD and SD. The leaves were found to yield almost the same amount of fresh weight during LD and SD, whereas the fresh weight of roots and stem plus leaf petioles increased considerably during LD.

Stem plus leaf petioles represents as much as 30% of the total plant fresh weight. The leaves contribute with relatively less, viz. 25%. During SD, the leaves make up 33% and stem plus leaf petioles 23% of the total plant fresh weight. There are no differences between SD and LD in the contribution of the roots to the plant fresh weight calculated in per cent. This is confirmed by the unchanged shoot:root weight ratio which is 1.22 under LD





and 1.24 under SD. The situation is almost the same for the dry weight, except that the leaves, during both photoperiods, yield a larger amount of dry weight than stem plus leaf petioles.

Spinacia plants grown under LD show a marked increment in the amount of fresh weight and dry weight in all plant parts as compared with Spinacia plants subjected to SD (Figure 2). The leaves contribute to the largest amount of fresh weight per plant, 48% under LD and 62% under SD conditions. The roots represent 35% of the total fresh weight under LD but only 16% under SD. This is also reflected in the shoot:root ratio which is 1.8 under LD and 5.0 under SD conditions.

The percentage contribution of the different plant parts to the amount of dry weight is, during both photoperiods, almost the same as for fresh weight.

#### Distribution of various nitrogenous fractions in Urtica plants during different photoperiods

##### Protein nitrogen

During both photoperiods, the amount of protein nitrogen per gram dry weight in Urtica (Figure 3 A) is highest in young leaves and lowest in the stem. The content of protein nitrogen per gram dry weight in old leaves is higher in SD, which indicates that the senescence is slower under SD conditions. The protein nitrogen content per dry weight of the other plant parts, except in the stem, increases slightly under LD conditions.

The total amount of protein nitrogen per plant is higher under LD than under SD conditions. Old leaves yield more protein in SD than in LD, whereas the opposite is true for young leaves. The amount of protein nitrogen in the stem and in the roots increases markedly with extended photoperiod (Figure 4 A).



### Soluble non-protein nitrogen

Young leaves have the largest amount of soluble nitrogen per gram dry weight in both LD and SD (Figure 3 B). The content in the stem is slightly higher in SD, whereas middle leaves exhibit a higher content in LD. No effect of the photoperiod on the total amount of soluble nitrogen per plant was observed (Figure 4 B).

### Nitrate nitrogen

In leaf tissue, the level of nitrate nitrogen per gram dry weight is very low in both LD and SD (Figure 3 C). As expected, the highest content was found in the stem, being the transport organ for non-reduced nitrogen. The nitrate contents in young leaves as well as roots are higher under LD conditions. The same holds for the total content of nitrate nitrogen per plant part (Figure 4 C).

### Ammonium nitrogen

The content of ammonium nitrogen is very low in all tissues of the plant in both LD and SD. Almost the same pattern is observed, whether the content of ammonium nitrogen is expressed per gram dry weight or per plant part (Figures 3 D and 4 D).

### Distribution of various nitrogenous fractions in Spinacia plants during different photoperiods

#### Protein nitrogen

All the different plant parts examined were found to have a higher amount of protein nitrogen per gram dry weight in SD as compared with LD (Figure 5 A). The highest content was observed in young leaves and the lowest in the stem under both LD and SD conditions. On the other hand, plants subjected to LD are superior as protein producers to plants subjected to SD (Figure 6 A).



### Soluble non-protein nitrogen

No significant differences between SD and LD were observed as to the content of soluble nitrogen per gram dry weight. This applies to all the different plant parts (Figure 5 B).

The total amount of soluble nitrogen per plant is only slightly higher in LD (Figure 6 B), which is due to a higher level in young leaves.

### Nitrate nitrogen

Figure 5 C shows that the content of nitrate nitrogen per gram dry weight in the leaves and the stem is higher in SD. The highest value is obtained for the stem in SD. The content in the roots is almost equal in LD and SD. Figure 6 C shows that the content of nitrate nitrogen expressed per plant part is very similar in SD and LD.

### Ammonium nitrogen

The content of ammonium per gram dry weight found in the tissue of different plant parts is very low in both LD and SD (Figure 5 D). Under SD conditions, a higher value was established for stem and roots as compared with LD conditions. When data are expressed per plant part, very low, similar values are observed in LD and SD (Figure 6 D).

### Enzyme activity in different parts of Urtica plants grown under LD and SD conditions

#### Nitrate reductase (NR) activity

No activity of the enzyme was obtained with in vitro assay. To preserve enzyme activity, it is necessary to include mercaptoethanol in the extraction medium. The harmful effect of mercaptoethanol on the NR activity in Urtica has earlier been shown (Welandar 1978). On the other hand, successful determinations of NR activity were recorded for both leaves and roots with in vivo assay. NR activity expressed per mg dry weight and hour is highest in the middle leaves but tends to decrease once the leaf has reached its





full size (Figure 7 C). Leaves of all the different age groups exhibited higher NR activity than roots. Plants subjected to LD were found to have a higher level of NR activity in old and middle leaves as well as roots.

The total NR activity expressed per plant (Figure 8 C) also has a higher level in leaves and roots under LD conditions. It is evident that despite the low activity recorded when expressed per gram dry weight, the roots contribute considerably to the enzymatic potential of the plant.

#### Glutamate dehydrogenase (GDH) activity

Assays to determine GDH showed that plants grown under LD conditions exhibit a slightly higher specific activity especially in old leaves and roots, than plants subjected to SD (Figure 7 D). Increased activity with leaf age was also recorded in LD. The total activity per plant is much higher in LD. It is also evident that roots contribute more than other plant parts to the total activity recovered from the plant in both LD and SD (Figure 8 A).

#### Aspartate aminotransferase (GOT) activity

The highest activity of GOT was found in roots in both LD and SD (Figures 7 B and 8 B). In SD, the distribution of the total GOT activity between leaves and roots is similar, whereas in LD the GOT activity was found to be slightly higher in the roots. In other respects, there is no effect of variations in the photoperiodic length.

#### Alanine aminotransferase (GPT) activity

Figure 7 A shows that the specific GPT activity increases markedly in the leaves under LD conditions. A slight increase with leaf age is also obtained in LD.

The total GPT activity in leaves and roots is much higher in LD than in SD (Figure 8 D). Among leaves of different age groups, the middle leaves were found to contribute most to the total activity. The total GPT activity is distributed almost equally among roots and leaves in both LD and SD.





## Enzyme activity in different parts of the Spinacia plants grown under LD and SD conditions

### Nitrate reductase (NR) activity

Measurable NR activity was obtained in leaves from spinach with both in vivo and in vitro assays. The levels of NR activity obtained with in vitro assay exceeded in vivo NR activity in middle and young leaves (data not shown). NR-activity in the roots was obtained with in vivo assay, whereas no such activity was detected in extracts from roots. It is assumed that failure to extract the enzyme is due to an NR inhibitor liberated during the extraction procedure. To test this assumption, an NR inhibitor assay was conducted. To a specified amount of an active NR preparation from leaves were added varying amounts of root extract and the degree of the NR activity inhibition was recorded. From the data shown in Table 2 it is evident that within the measured range the degree of inhibition in per cent is almost linear.

NR inhibitors from corn roots (Wallace 1973) and rice roots (Kadam et al. 1974) have previously been isolated. Plants grown in SD show a higher level of NR activity per gram dry weight and hour in young leaves and roots (Figure 9 C). On the other hand, the total NR activity in these plant parts is higher in LD than in SD (Figure 10 C). The total activity is higher in leaves than in roots in both LD and SD but the contribution of the roots should not be neglected.

### Glutamate dehydrogenase (GDH) activity

In Spinacia, specific GDH activity is much higher in roots than in leaves in both LD and SD. Increased activity with leaf age was also noted (Figure 9 D). Roots being strongly affected by photoperiodic variations showed increased specific activity in LD. The total GDH activity per plant part, as shown in Figure 10 D, indicates that the activity recovered from roots and young leaves is considerably higher in LD than in SD. Under SD conditions, the leaves contribute most to the total activity recovered from the plant, whereas in LD the roots contribute most to GDH activity.



### Aspartate aminotransferase (GOT) activity

The influence of extended photoperiod on specific GOT activity in various plant parts appears from Figure 9 B. In leaves, no significant differences in the specific GOT activity were observed between LD and SD, whereas in the roots a slight increase was noted. The photoperiodic effect on the GOT activity in the roots can also be observed when the activity is expressed per plant part (Figure 10 B). In SD, the contribution of the roots to the enzymatic potential is relatively low.

### Alanine aminotransferase (GPT) activity

Figure 9 A shows that extended photoperiod increases the specific GPT activity in all the different plant parts examined. The total GPT activity expressed per plant part (Figure 10 A) shows a marked increase only in the roots. Among leaves of different age groups, middle leaves contribute most to the total activity in both LD and SD and only a minor part of the total activity is located in the roots, especially in SD.



## Discussion

In tables 3 and 4, the most important data are summarized and subjected to statistical analyses. The data are expressed as the difference ( $\Delta$ ) between the mean values of the two cultivations obtained in LD and SD.

$S_{\Delta}$  denotes the standard error of the differences between the means and is calculated as follows  $S_{\Delta} = \sqrt{S_{m_1}^2 + S_{m_2}^2}$   $S_m$  = standard error or mean.

Positive values denote increase in LD, whereas negative values denote increase in SD. It will be seen that an extension of the daylength increases the fresh weight and dry weight as well as the protein content in both *Urtica* and *Spinacia*. In *Urtica*, the additional dry matter produced in LD is present in stem plus leaf petioles and root. In the root, the amount of protein per g d.wt. also increases in LD, resulting in a much higher protein production. In the stem, no changes in the amount of protein per g d.wt. are observed. However, because of the increased dry matter production, the total amount of protein is higher in LD. Old leaves produce more protein per g d.wt. in SD, which indicates that the senescence starts earlier in LD. On the other hand, the other leaf fractions produce more protein per g d.wt. in LD, resulting in a higher total amount of protein in the leaves in LD as compared with SD. In *Spinacia*, all the different plant parts produce more protein per g d.wt. in SD. Nonetheless, a considerably higher amount of protein is obtained in leaves and roots in LD. This is due to a substantial increase in root growth and to a higher dry matter production in the roots and in young and middle leaves.

A positive correlation between the level of NR activity and the protein content of the plants was demonstrated by Croy and Hageman (1970) and Liu and Hadley (1971). In *Urtica* subjected to LD, a higher total amount of protein in all leaves and in the roots is accompanied by a higher level of NR activity, whereas in *Spinacia* subjected to LD, a positive correlation in this respect is obtained only in the roots. On the other hand, comparisons between the amount of protein per g d.wt. and NR activity per g d.wt. in the





different plant parts have not given any consistent results in *Urtica*, a positive correlation was obtained in middle leaves and roots under LD conditions, whereas in *Spinacia* such a correlation was found in young leaves and roots under SD conditions. It has been shown that the formation, activation and stability of NR depend on the availability of nitrate (Hewitt 1975). In *Urtica*, much more nitrate per g d.wt. was found in the roots in LD, which may explain the higher level of NR activity in the roots during this photoperiod. In *Spinacia*, on the other hand, the increased level of NR activity per g d.wt. in the roots that was observed in SD is not accompanied by an accumulation of nitrate. This is probably due to the increased transport of nitrate from the root to the shoot indicated by the higher nitrate content in stem plus leaf petioles in SD. Differences between other species as to their ability to transport nitrate from the root to the shoot have earlier been demonstrated by Radin (1978). Until recently, it was assumed that GDH was the most important enzyme in ammonium assimilation in higher plants. Owing to the high  $K_m$  value of GDH for ammonium ions, it is now suggested that GDH plays a limited part in the pathway of ammonium assimilation (Mifflin and Lea 1977). Most of the nitrate is reduced in the leaves in both *Urtica* and *Spinacia*. The highest specific activity of GDH is obtained in the roots. In other species as well, the GDH activity obtained in the roots was much higher than that obtained in the leaves (Wallace 1973, Pšenáková et al. 1976, Pahlich and Joy 1971). Increased specific activity of GDH under LD conditions was also recorded mainly for the roots in both *Urtica* and *Spinacia*. These results are consistent with the hypothesis that GDH is mostly concerned with glutamate degradation. Givan (1978) suggested that another important role of GDH resides in disposing of high ammonium levels. On the other hand, Lewis has shown that plants supplied with only ammonium as nitrogen source in the nutrient solution do not exhibit increased GDH activity (personal communication). The role of GDH is still disputed and further experiments must be carried out in order to solve this issue.



As earlier shown by Welander (1974), the specific activity of GPT in leaves from *Urtica* increases under LD conditions, whereas very small changes are observed in the specific activity of GOT. Hedley and Stoddart (1971) and Thomas (1974) demonstrated a close relationship between chlorophyll content and GPT activity. In *Urtica*, the raised chlorophyll content obtained in leaves of different age groups in LD (Table 1) might be an explanation of the increased specific activity of GPT in LD. It should be noted that in LD the old leaves having the highest chlorophyll content show the highest GPT activity, whereas in SD the same correlation is found in middle leaves.

In *Urtica*, the specific activity of GOT and GPT in the roots does not change during the extended photoperiod. On the other hand, the total enzyme activity increases during LD because of the increased amount of protein. In *Spinacia*, the specific activity of GOT and GPT and the total enzyme activity substantially increase in the roots under LD conditions.



Table 1. Changes in morphological parameters in *Urtica dioica* and *Spinacia oleracea* subjected to either SD or LD conditions, and changes in the chlorophyll content in leaves of different age groups from *Urtica*. The data are the means of two separate cultivations and  $\pm$  denotes the difference between the cultivations.

|                                                   | Urtica dioica  |                                                                  | Spinacia oleracea |                |
|---------------------------------------------------|----------------|------------------------------------------------------------------|-------------------|----------------|
|                                                   | LD             | SD                                                               | LD                | SD             |
| Total leaf lamina <sup>r</sup> (cm <sup>2</sup> ) | 16.7 $\pm$ 20  | 146 $\pm$ 28                                                     | 88 $\pm$ 4        | 52 $\pm$ 10    |
| Leaf number on the main shoot                     | 14.6 $\pm$ 1.0 | 12.0 $\pm$ 1.1<br>(several small leaves on the lateral branches) | 11.6 $\pm$ 1.5    | 8.9 $\pm$ 1.2  |
| Internode length (cm)                             | 8.2 $\pm$ 0.8  | 5.1 $\pm$ 0.7                                                    | -                 | -              |
| Shoot length of the main axis (cm)                | 48.6 $\pm$ 9.2 | 28.8 $\pm$ 4.5                                                   | 8                 | -<br>(rosette) |
| Root length (cm)                                  | 15.1 $\pm$ 1.5 | 16.1 $\pm$ 1.5                                                   | 19.2 $\pm$ 3.0    | 14.4 $\pm$ 1.8 |

| Chlorophyll content |                   |                   |
|---------------------|-------------------|-------------------|
| mg per g f.wt       |                   |                   |
|                     | LD                | SD                |
| Old leaves          | 0.340 $\pm$ 0.004 | 0.262 $\pm$ 0.008 |
| Middle leaves       | 0.334 $\pm$ 0.020 | 0.286 $\pm$ 0.007 |
| Young leaves        | 0.304 $\pm$ 0.004 | 0.183 $\pm$ 0.020 |





Table 2. Demonstration of a nitrate reductase inhibitor from *Spinacia* roots. Varying amounts of root extract were added to a specified amount of active nitrate reductase preparation from *Spinacia* leaves and the degree of nitrate reductase inhibition was recorded.

| ml root extract added to<br>0.5 ml active NR preparation<br>from leaves | NR-activity (in vitro assay)<br>$\mu\text{mol NO}_2^-$ per g f. wt. h<br>% inhibition |
|-------------------------------------------------------------------------|---------------------------------------------------------------------------------------|
| 0                                                                       | 3.51 0                                                                                |
| 0.1                                                                     | 3.45 2                                                                                |
| 0.2                                                                     | 3.08 12                                                                               |
| 0.5                                                                     | 2.73 22.3                                                                             |
| 1.0                                                                     | 2.03 42.2                                                                             |



Table 3. Summary of the most important results for *Urtica dioica*. The data are subjected to statistical analyses.

$\Delta$  = the difference between the mean values of the two cultivations obtained in LD and SD.

$S_{\Delta}$  = standard error of the differences between the means. \* act. = activity

*Urtica dioica*

| Category                    | All leaves                              | Leaves                                  |                                         |                                        | Stem + leaf petioles                    | Root                                   | Category                  | Leaves                                  |                                         |                                         | Stem                                    | Root                                   |
|-----------------------------|-----------------------------------------|-----------------------------------------|-----------------------------------------|----------------------------------------|-----------------------------------------|----------------------------------------|---------------------------|-----------------------------------------|-----------------------------------------|-----------------------------------------|-----------------------------------------|----------------------------------------|
|                             |                                         | old                                     | middle                                  | young                                  |                                         |                                        |                           | old                                     | middle                                  | young                                   |                                         |                                        |
| Fresh weight g/plant in     | $\Delta = 0.16$<br>$S_{\Delta} = 0.30$  | $\Delta = 0.01$<br>$S_{\Delta} = 0.11$  | $\Delta = 0.03$<br>$S_{\Delta} = 0.09$  | $\Delta = 0.13$<br>$S_{\Delta} = 0.11$ | $\Delta = 1.59$<br>$S_{\Delta} = 0.21$  | $\Delta = 1.50$<br>$S_{\Delta} = 0.31$ |                           |                                         |                                         |                                         |                                         |                                        |
| Dry weight g/plant in       | $\Delta = 0.02$<br>$S_{\Delta} = 0.11$  | $\Delta = -0.01$<br>$S_{\Delta} = 0.03$ | $\Delta = -0.01$<br>$S_{\Delta} = 0.03$ | $\Delta = 0.04$<br>$S_{\Delta} = 0.03$ | $\Delta = 0.18$<br>$S_{\Delta} = 0.03$  | $\Delta = 0.19$<br>$S_{\Delta} = 0.01$ |                           |                                         |                                         |                                         |                                         |                                        |
| Protein mg/plant in         | $\Delta = 1.42$<br>$S_{\Delta} = 0.19$  | $\Delta = -0.85$<br>$S_{\Delta} = 0.09$ | $\Delta = 0.29$<br>$S_{\Delta} = 0.19$  | $\Delta = 1.98$<br>$S_{\Delta} = 0.14$ | $\Delta = 2.10$<br>$S_{\Delta} = 0.51$  | $\Delta = 7.10$<br>$S_{\Delta} = 1.73$ | Protein mg/g d.wt         | $\Delta = -3.10$<br>$S_{\Delta} = 0.43$ | $\Delta = 1.80$<br>$S_{\Delta} = 0.68$  | $\Delta = 2.00$<br>$S_{\Delta} = 1.10$  | $\Delta = -0.90$<br>$S_{\Delta} = 1.94$ | $\Delta = 4.60$<br>$S_{\Delta} = 2.80$ |
| NO <sub>3</sub> mg/plant in | $\Delta = 0.69$<br>$S_{\Delta} = 0.38$  | $\Delta = 0.13$<br>$S_{\Delta} = 0.17$  | $\Delta = 0.22$<br>$S_{\Delta} = 0.18$  | $\Delta = 0.28$<br>$S_{\Delta} = 0.04$ | $\Delta = 2.04$<br>$S_{\Delta} = 1.05$  | $\Delta = 1.82$<br>$S_{\Delta} = 0.13$ | NO <sub>3</sub> mg/g d.wt | $\Delta = 0.70$<br>$S_{\Delta} = 0.91$  | $\Delta = -1.60$<br>$S_{\Delta} = 0.67$ | $\Delta = 1.70$<br>$S_{\Delta} = 0.25$  | $\Delta = 2.30$<br>$S_{\Delta} = 3.1$   | $\Delta = 2.60$<br>$S_{\Delta} = 0.27$ |
| NR * act./plant in          | $\Delta = 0.99$<br>$S_{\Delta} = 0.14$  | $\Delta = 0.22$<br>$S_{\Delta} = 0.09$  | $\Delta = 0.43$<br>$S_{\Delta} = 0.20$  | $\Delta = 0.34$<br>$S_{\Delta} = 0.15$ | Root                                    |                                        | NR act./g d.wt            | $\Delta = -0.97$<br>$S_{\Delta} = 0.38$ | $\Delta = 1.36$<br>$S_{\Delta} = 0.78$  | $\Delta = 0.34$<br>$S_{\Delta} = 1.10$  | Root                                    |                                        |
| GDH act./plant in           | $\Delta = 11.80$<br>$S_{\Delta} = 1.66$ | $\Delta = 2.20$<br>$S_{\Delta} = 0.28$  | $\Delta = 6.90$<br>$S_{\Delta} = 1.30$  | $\Delta = 2.70$<br>$S_{\Delta} = 0.65$ | $\Delta = 105.5$<br>$S_{\Delta} = 20.2$ |                                        | GDH specific activity     | $\Delta = 0.94$<br>$S_{\Delta} = 0.42$  | $\Delta = 0.77$<br>$S_{\Delta} = 0.52$  | $\Delta = 0.51$<br>$S_{\Delta} = 0.33$  | $\Delta = 11.00$<br>$S_{\Delta} = 4.95$ |                                        |
| GPT act./plant in           | $\Delta = 122.4$<br>$S_{\Delta} = 34.4$ | $\Delta = 27.4$<br>$S_{\Delta} = 11.1$  | $\Delta = 60.9$<br>$S_{\Delta} = 15.7$  | $\Delta = 34.0$<br>$S_{\Delta} = 8.85$ | $\Delta = 72.9$<br>$S_{\Delta} = 24.5$  |                                        | GPT specific activity     | $\Delta = 7.56$<br>$S_{\Delta} = 0.96$  | $\Delta = 5.90$<br>$S_{\Delta} = 1.59$  | $\Delta = 5.44$<br>$S_{\Delta} = 2.86$  | $\Delta = 2.00$<br>$S_{\Delta} = 5.41$  |                                        |
| GOT act./plant in           | $\Delta = 12.3$<br>$S_{\Delta} = 28.1$  | $\Delta = -2.4$<br>$S_{\Delta} = 10.8$  | $\Delta = 11.5$<br>$S_{\Delta} = 15.4$  | $\Delta = 3.20$<br>$S_{\Delta} = 4.49$ | $\Delta = 29.7$<br>$S_{\Delta} = 20.0$  |                                        | GOT specific activity     | $\Delta = 1.26$<br>$S_{\Delta} = 0.94$  | $\Delta = 0.67$<br>$S_{\Delta} = 0.54$  | $\Delta = -0.23$<br>$S_{\Delta} = 0.94$ | $\Delta = 0.63$<br>$S_{\Delta} = 1.94$  |                                        |



**Table 4.** Summary of the most important results for *Spinacia oleracea*. The data are subjected to statistical analyses.

$\Delta$  = the difference between the mean values of the two cultivations obtained in LD and SD.

$S_{\Delta}$  = standard error of the differences between the means. \* act. = activity

*Spinacia oleracea*

| Category                    | All leaves                              | Leaves                                  |                                        |                                        | Stem + leaf petioles                   | Root                                   | Category                  | Leaves                                  |                                         |                                         | Stem                                   | Root                                  |
|-----------------------------|-----------------------------------------|-----------------------------------------|----------------------------------------|----------------------------------------|----------------------------------------|----------------------------------------|---------------------------|-----------------------------------------|-----------------------------------------|-----------------------------------------|----------------------------------------|---------------------------------------|
|                             |                                         | old                                     | middle                                 | young                                  |                                        |                                        |                           | old                                     | middle                                  | young                                   |                                        |                                       |
| Fresh weight g/plant in     | $\Delta = 0.82$<br>$S_{\Delta} = 0.22$  | $\Delta = 0.10$<br>$S_{\Delta} = 0.09$  | $\Delta = 0.34$<br>$S_{\Delta} = 0.08$ | $\Delta = 0.38$<br>$S_{\Delta} = 0.05$ | $\Delta = 0.24$                        | $\Delta = 1.92$<br>$S_{\Delta} = 0.20$ |                           |                                         |                                         |                                         |                                        |                                       |
| Dry weight g/plant in       | $\Delta = 0.11$<br>$S_{\Delta} = 0.05$  | $\Delta = 0.01$<br>$S_{\Delta} = 0.02$  | $\Delta = 0.04$<br>$S_{\Delta} = 0.02$ | $\Delta = 0.06$<br>$S_{\Delta} = 0.01$ | $\Delta = 0.05$                        | $\Delta = 0.24$<br>$S_{\Delta} = 0.03$ |                           |                                         |                                         |                                         |                                        |                                       |
| Protein mg/plant in         | $\Delta = 3.40$<br>$S_{\Delta} = 0.54$  | $\Delta = 0.36$<br>$S_{\Delta} = 0.21$  | $\Delta = 0.83$<br>$S_{\Delta} = 0.30$ | $\Delta = 2.93$<br>$S_{\Delta} = 0.07$ | $\Delta = 0.49$                        | $\Delta = 3.68$<br>$S_{\Delta} = 0.72$ | Protein mg/g d.wt         | $\Delta = -6.70$<br>$S_{\Delta} = 2.00$ | $\Delta = -3.70$<br>$S_{\Delta} = 1.56$ | $\Delta = -3.80$<br>$S_{\Delta} = 0.78$ | $\Delta = -2.5$                        | $\Delta = -5.1$<br>$S_{\Delta} = 2.2$ |
| NO <sub>3</sub> mg/plant in | $\Delta = 0.71$<br>$S_{\Delta} = 0.57$  | $\Delta = 0.26$<br>$S_{\Delta} = 0.14$  | $\Delta = 0.36$<br>$S_{\Delta} = 0.23$ | $\Delta = 0.05$<br>$S_{\Delta} = 0.14$ | $\Delta = 1.01$                        | $\Delta = 0.57$<br>$S_{\Delta} = 0.41$ | NO <sub>3</sub> mg/g d.wt | $\Delta = -2.80$<br>$S_{\Delta} = 1.91$ | $\Delta = -2.20$<br>$S_{\Delta} = 1.03$ | $\Delta = -2.90$<br>$S_{\Delta} = 2.50$ | $\Delta = -12.8$                       | $\Delta = -0.20$                      |
| NR act./plant in            | $\Delta = 0.31$<br>$S_{\Delta} = 0.42$  | $\Delta = 0.01$<br>$S_{\Delta} = 0.04$  | $\Delta = 0.16$<br>$S_{\Delta} = 0.30$ | $\Delta = 0.14$<br>$S_{\Delta} = 0.11$ | Root                                   |                                        | NR act./g d.wt            | $\Delta = -0.11$<br>$S_{\Delta} = 0.93$ | $\Delta = -0.01$<br>$S_{\Delta} = 0.64$ | $\Delta = -1.17$<br>$S_{\Delta} = 0.86$ | Root                                   |                                       |
| GDH act./plant in           | $\Delta = 14.4$<br>$S_{\Delta} = 11.23$ | $\Delta = -4.20$<br>$S_{\Delta} = 5.41$ | $\Delta = 6.00$<br>$S_{\Delta} = 5.84$ | $\Delta = 12.6$<br>$S_{\Delta} = 2.08$ | $\Delta = 81.3$<br>$S_{\Delta} = 10.4$ |                                        | GDH specific activity     | $\Delta = 4.2$<br>$S_{\Delta} = 5.0$    | $\Delta = 3.0$<br>$S_{\Delta} = 3.0$    | $\Delta = 2.5$<br>$S_{\Delta} = 2.0$    | $\Delta = 27.9$<br>$S_{\Delta} = 6.8$  |                                       |
| GPT act./plant in           | $\Delta = 8.70$<br>$S_{\Delta} = 32.6$  | $\Delta = -4.20$<br>$S_{\Delta} = 7.41$ | $\Delta = 6.70$<br>$S_{\Delta} = 19.0$ | $\Delta = 15.0$<br>$S_{\Delta} = 6.71$ | $\Delta = 11.4$<br>$S_{\Delta} = 1.8$  |                                        | GPT specific activity     | $\Delta = 5.20$<br>$S_{\Delta} = 1.75$  | $\Delta = 4.5$<br>$S_{\Delta} = 2.6$    | $\Delta = 2.8$<br>$S_{\Delta} = 1.9$    | $\Delta = 9.6$<br>$S_{\Delta} = 4.3$   |                                       |
| GOT act./plant in           | $\Delta = 1.80$<br>$S_{\Delta} = 16.2$  | $\Delta = -7.5$<br>$S_{\Delta} = 6.0$   | $\Delta = 6.40$<br>$S_{\Delta} = 10.7$ | $\Delta = 2.90$<br>$S_{\Delta} = 1.78$ | $\Delta = 40.2$<br>$S_{\Delta} = 4.6$  |                                        | GOT specific activity     | $\Delta = 4.10$<br>$S_{\Delta} = 4.90$  | $\Delta = 2.60$<br>$S_{\Delta} = 3.56$  | $\Delta = 1.2$<br>$S_{\Delta} = 2.13$   | $\Delta = 20.5$<br>$S_{\Delta} = 10.0$ |                                       |







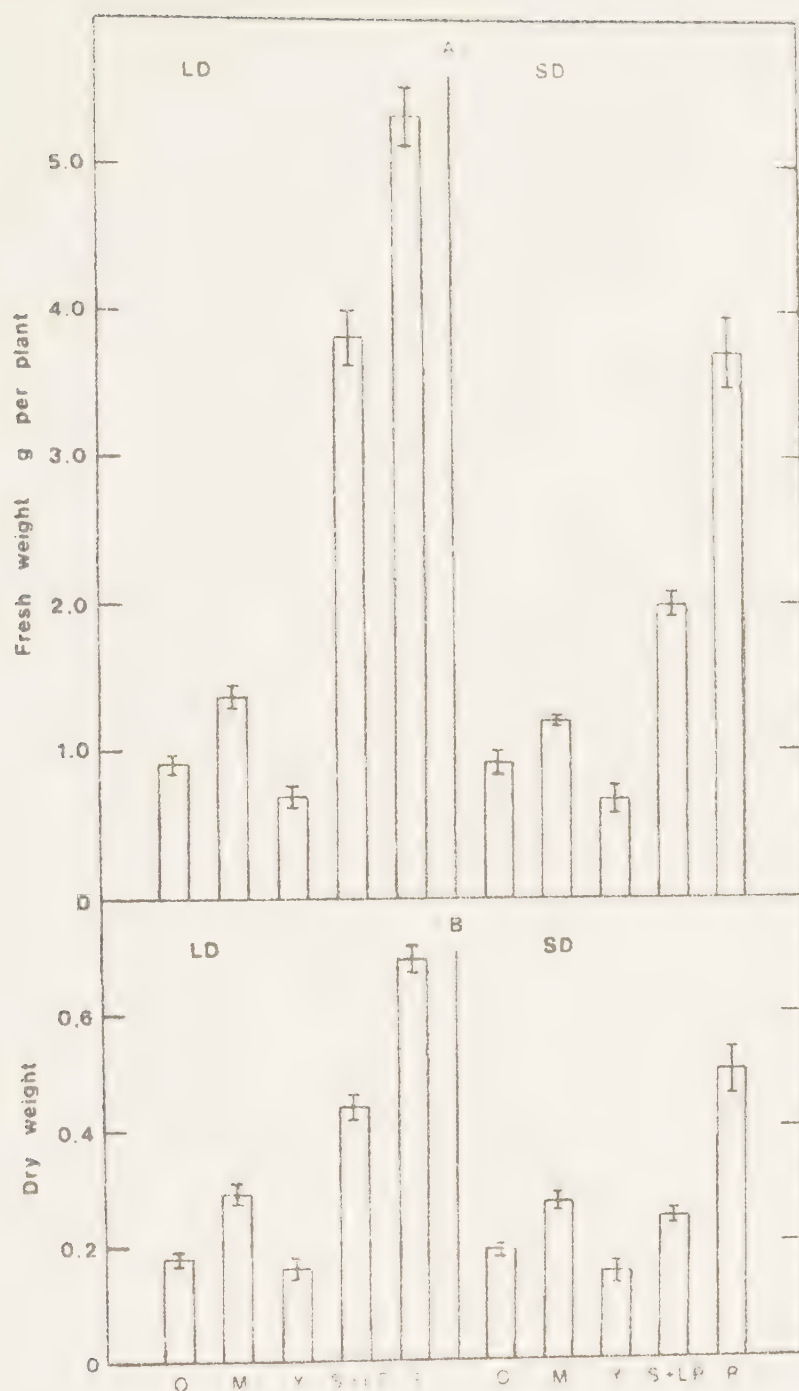


Figure 1. The influence of photoperiodic variation on the amount of fresh weight (A) and dry weight (B) in different plant parts from *Urtica dioica*. O = old leaves, M = middle leaves, Y = young leaves, S + LP = stem plus leaf petioles, and R = roots. Each staple represents a mean value. The vertical bars denote the difference between data obtained from two separate cultivations.



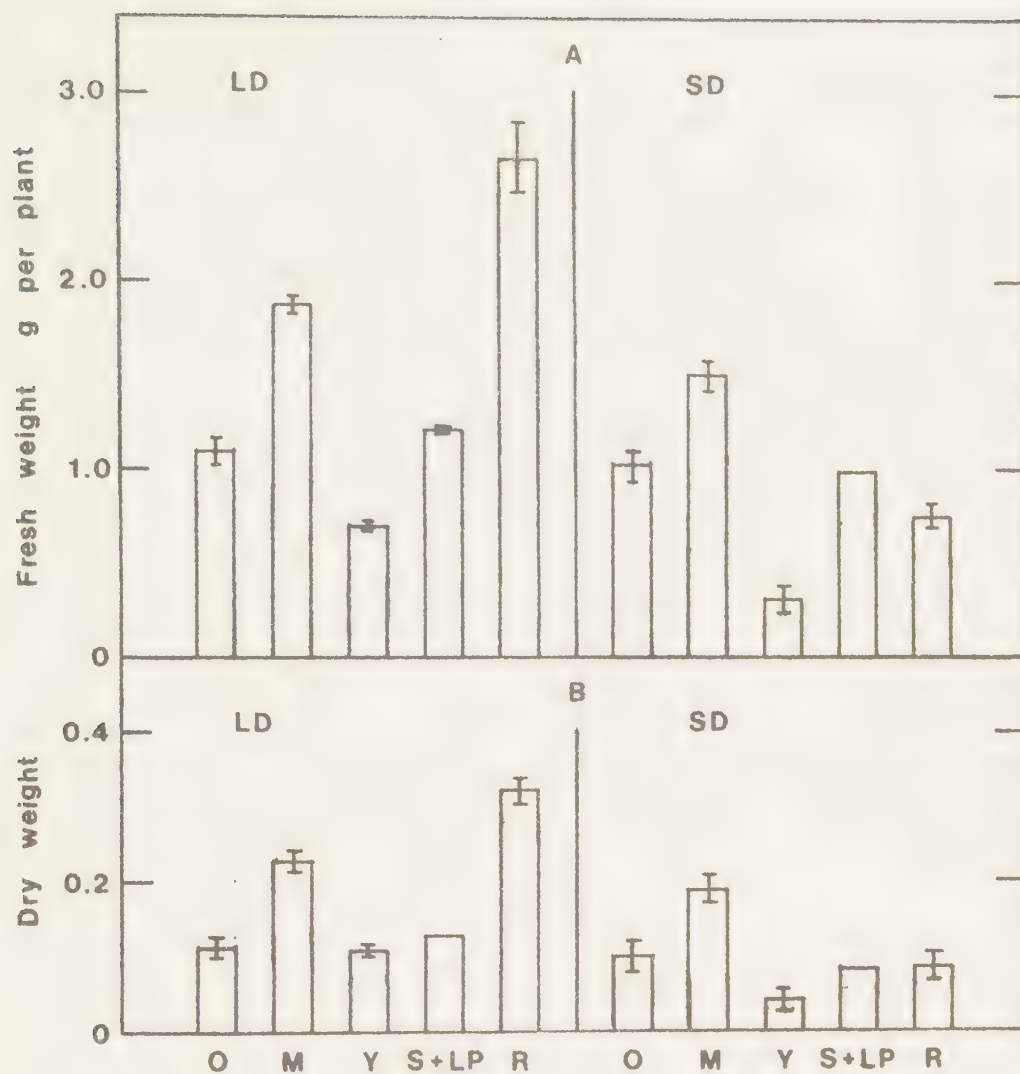


Figure 2. The influence of photoperiodic variation on the amount of fresh weight (A) and dry weight (B) in different plant parts from *Spinacia oleracea*. Data expressed as in figure 1.



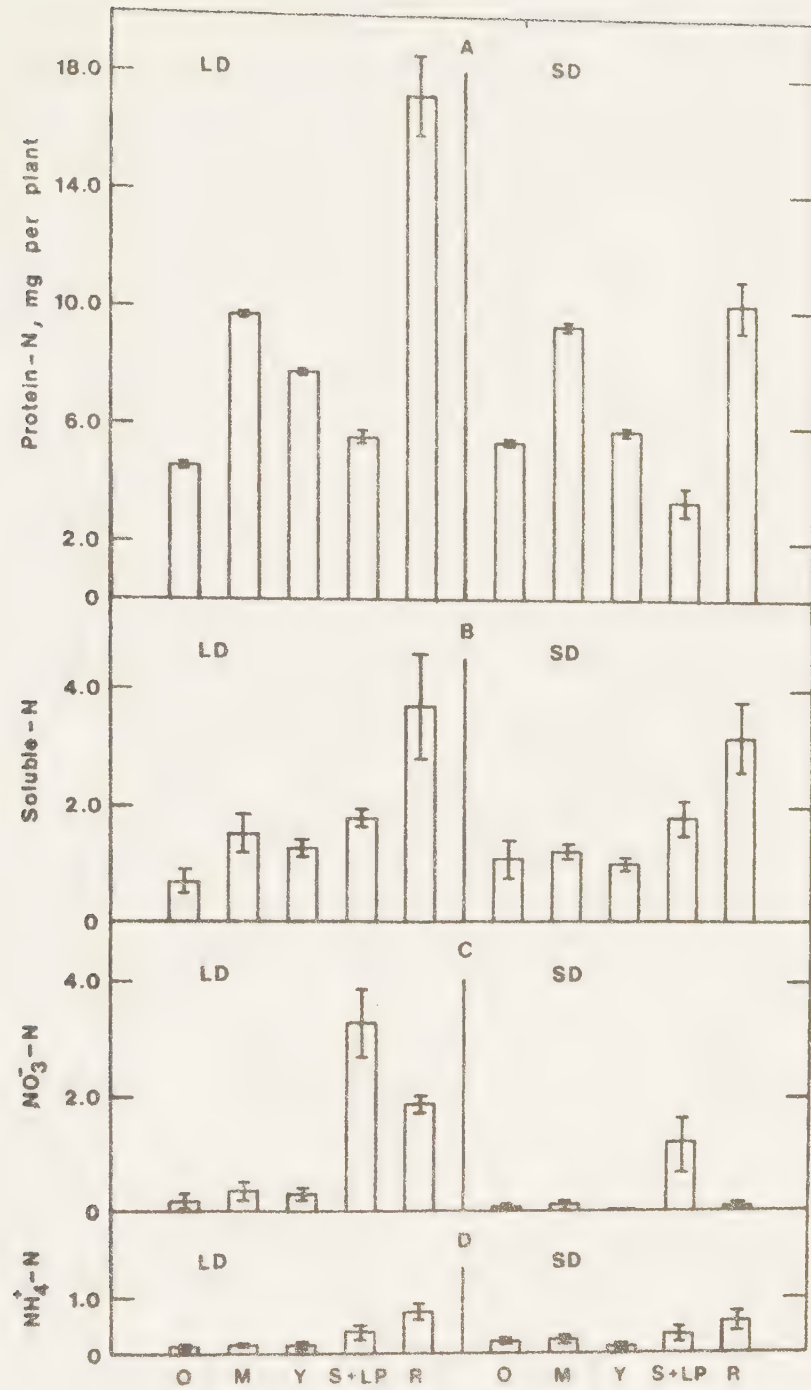


Figure 3. Influence of photoperiodic variation on the distribution of various nitrogenous fractions expressed as mg per g d.wt in different parts of *Urtica* plants. A: protein nitrogen, B: soluble non-protein nitrogen, C: nitrate nitrogen, and D: ammonium nitrogen. The different nitrogenous preparations are described under Materials and Methods. Otherwise, data expressed as in figure 1.



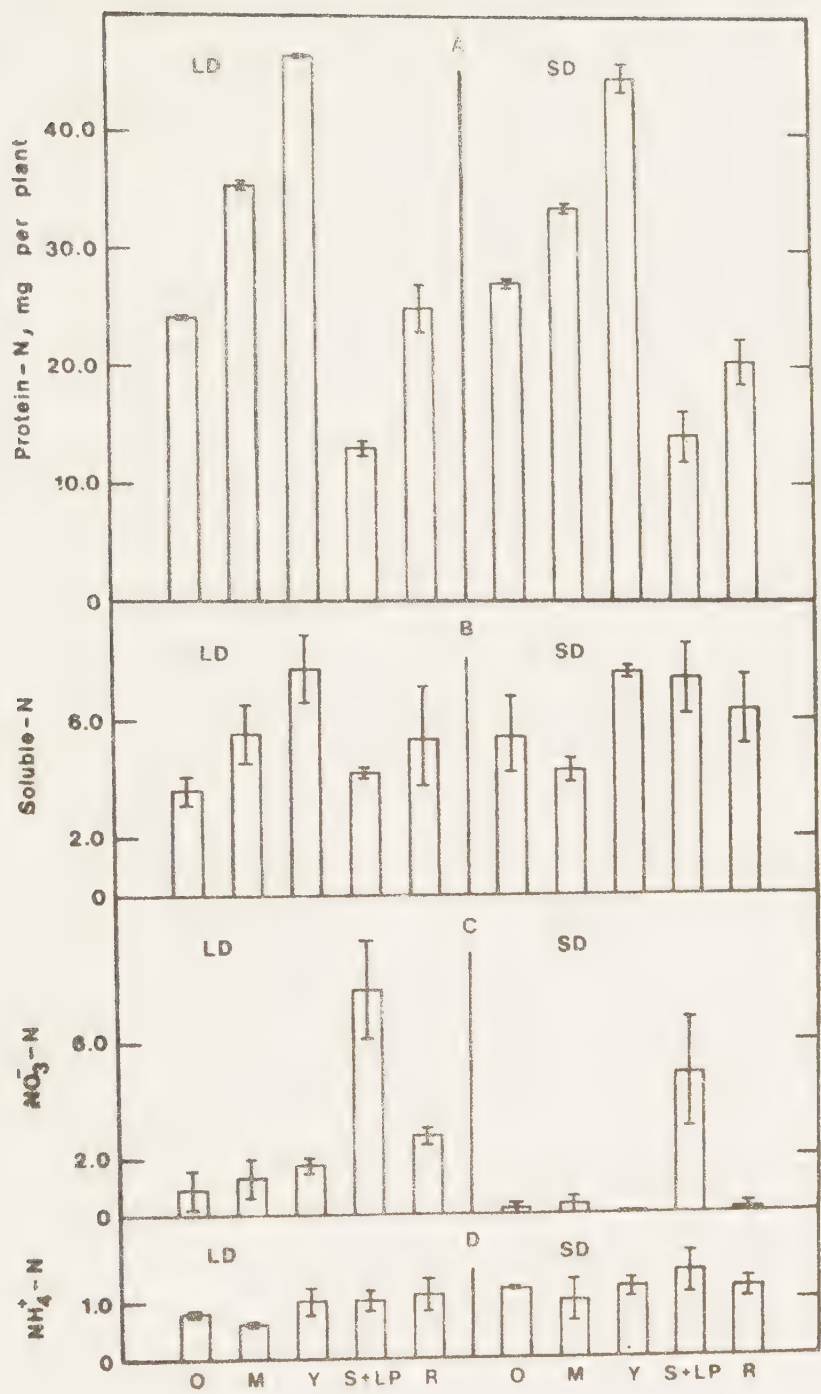


Figure 4. The same as in figure 3, except that data are expressed as mg per plant part.





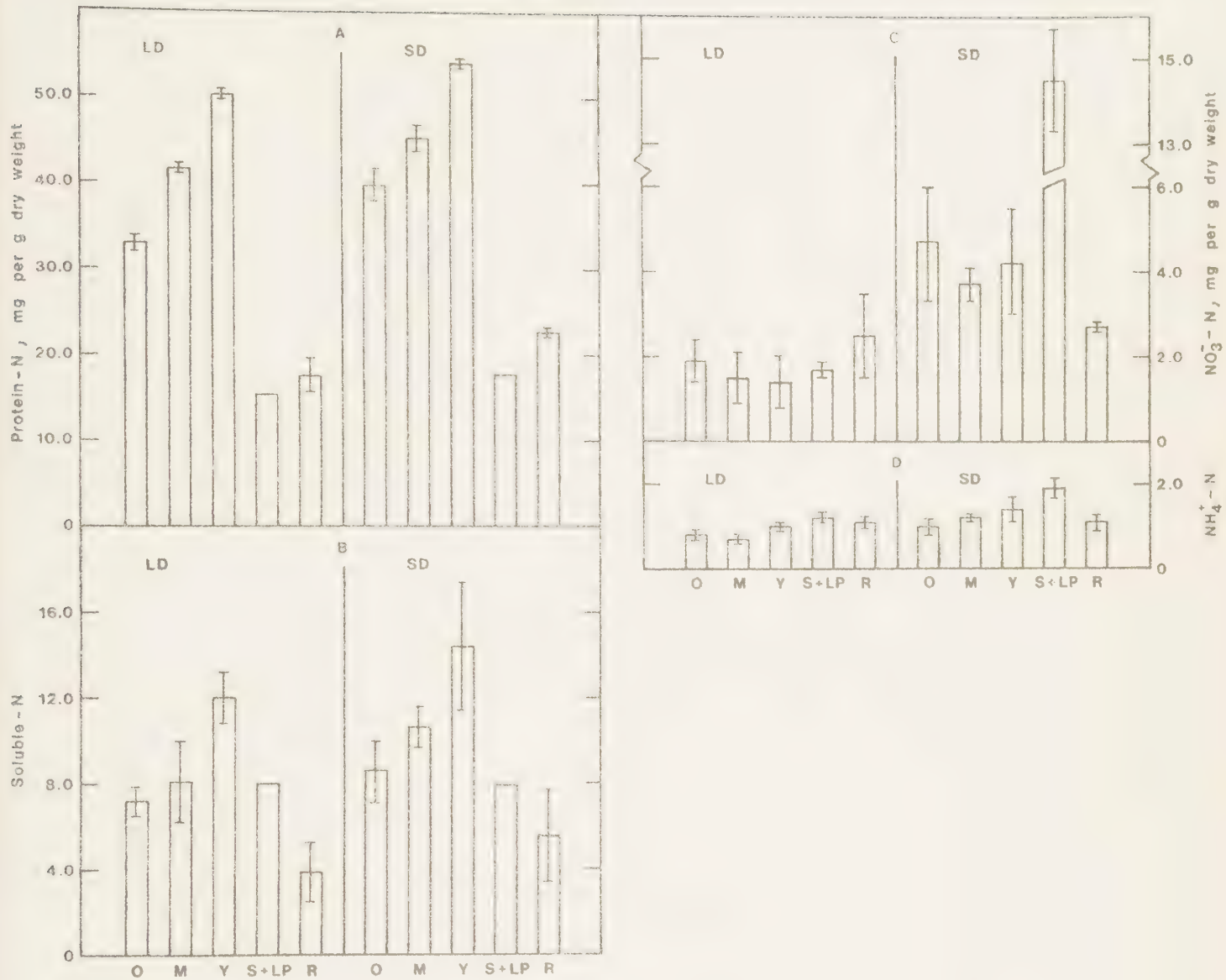


Figure 5. Influence of photoperiodic variation on the distribution of various nitrogenous fractions expressed as mg per g d.wt in different parts of *Spinacia* plants. A: protein nitrogen, B: soluble non-protein nitrogen, C: nitrate nitrogen, and D: ammonium nitrogen. The different nitrogenous preparations are described under Materials and Methods. Otherwise, data expressed as in figure 1.



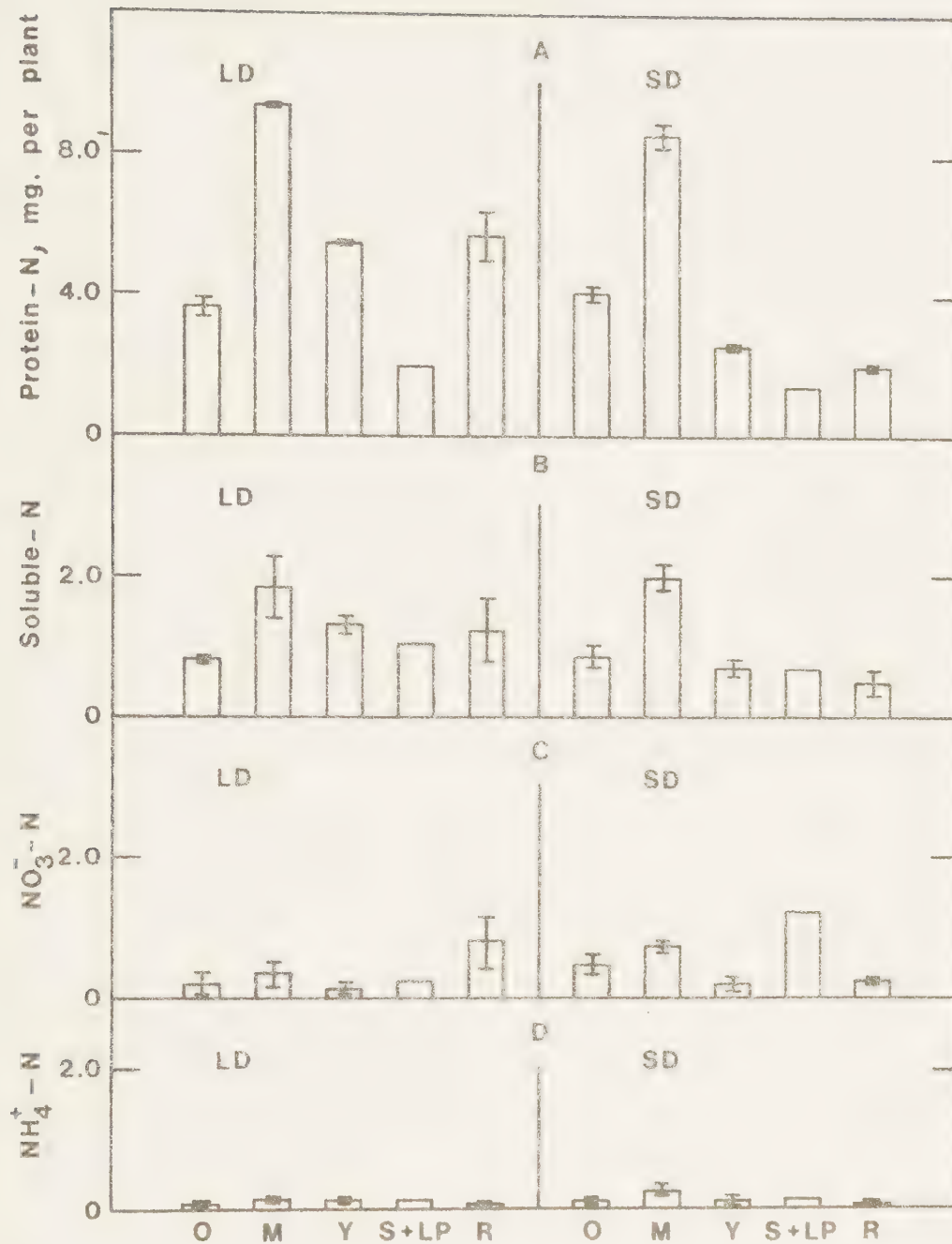


Figure 6. The same as in figure 5, except that data are expressed as mg per plant part.



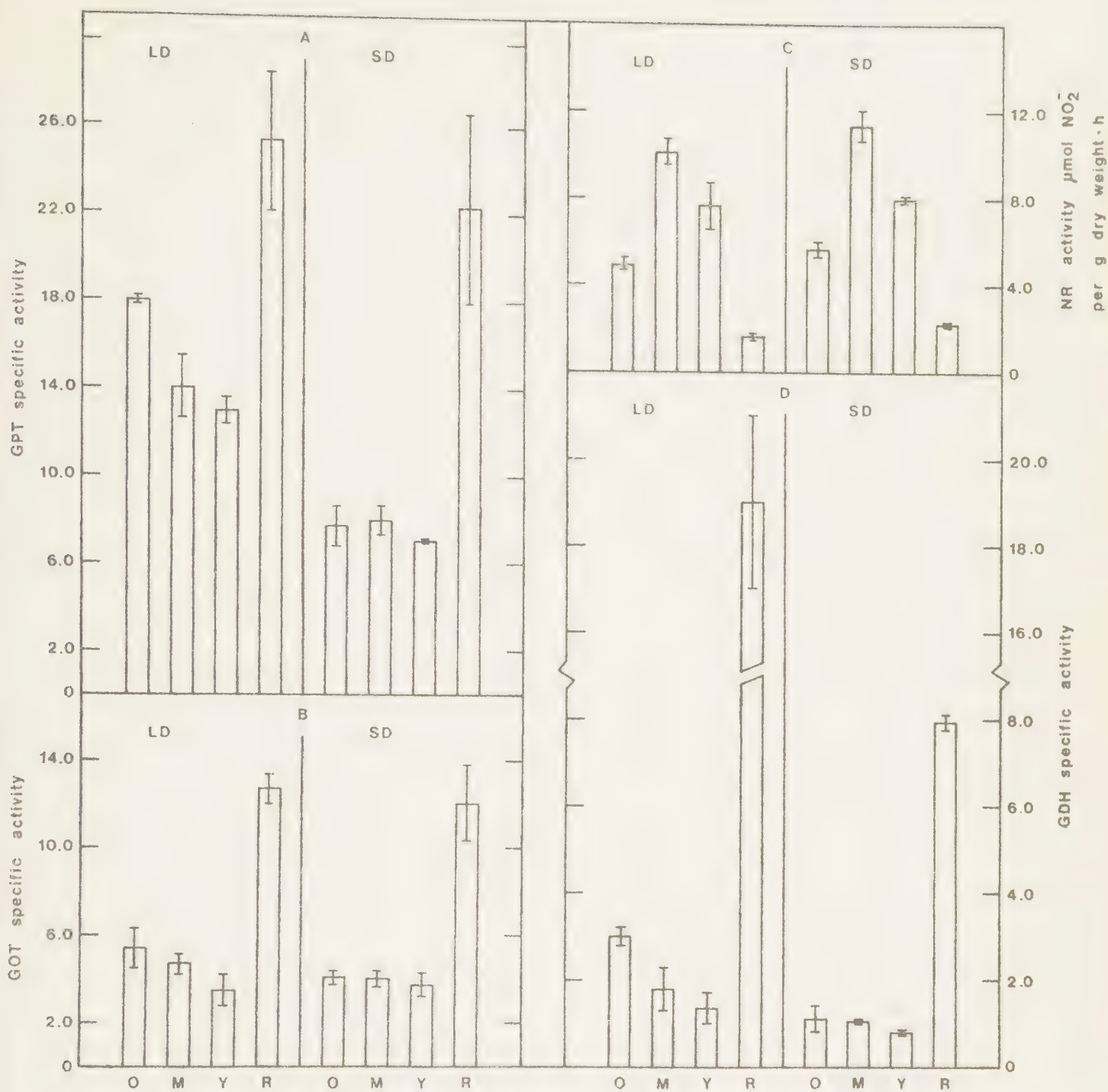


Figure 7. Enzyme activity in different parts of *Urtica* plants grown under LD and SD conditions.

A: GPT activity in  $\mu\text{mol}$  keto acid produced per h and mg protein,

B: GOT activity in  $\mu\text{mol}$  keto acid produced per h and mg protein,

C: NR activity in  $\mu\text{mol NO}_2^-$  per g d.wt and h, and

D: GDH activity in units per mg protein.

Otherwise, data expressed as in figure 1.





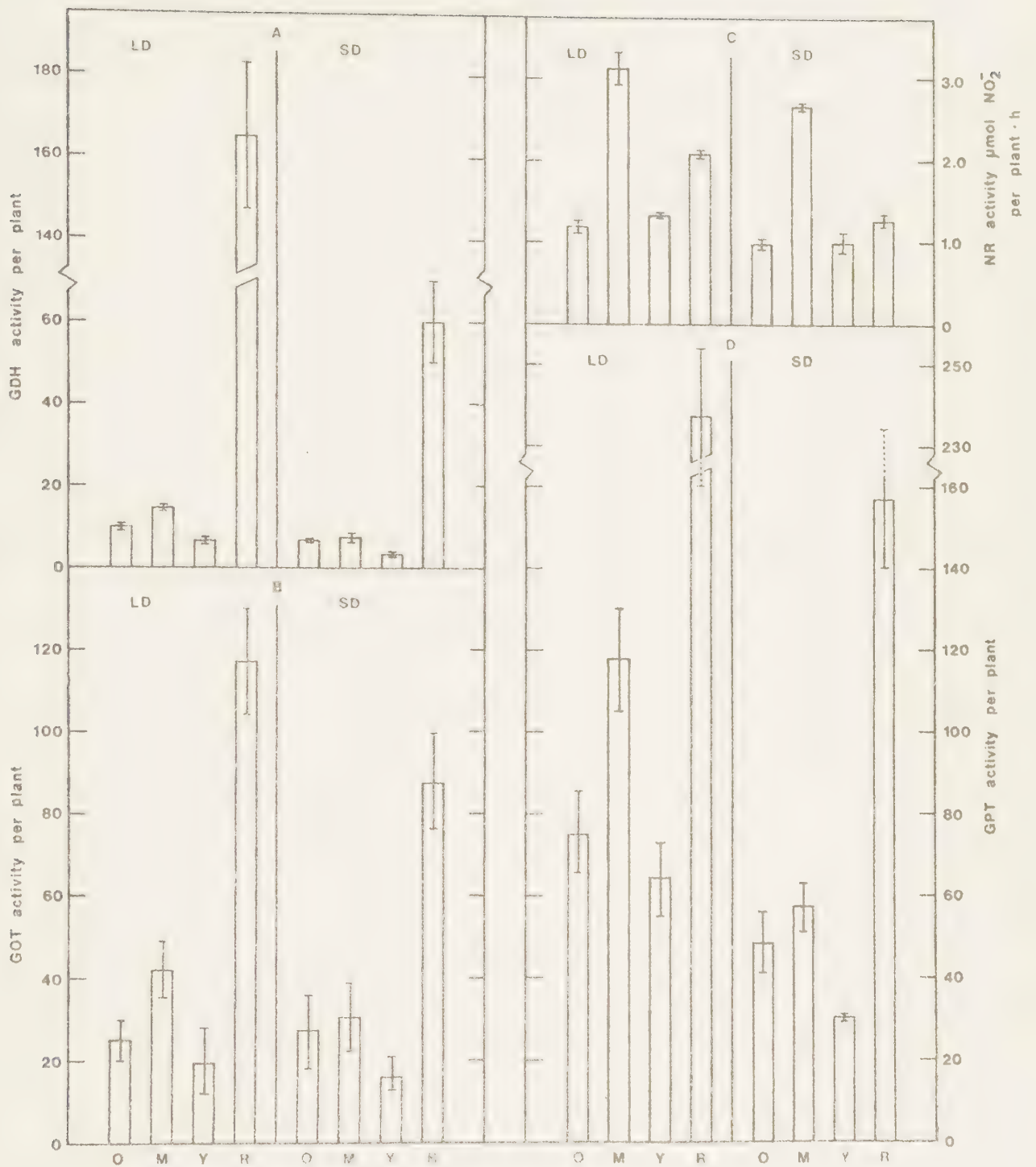


Figure 8. The same as in figure 7, except that enzyme activity is expressed per plant part.



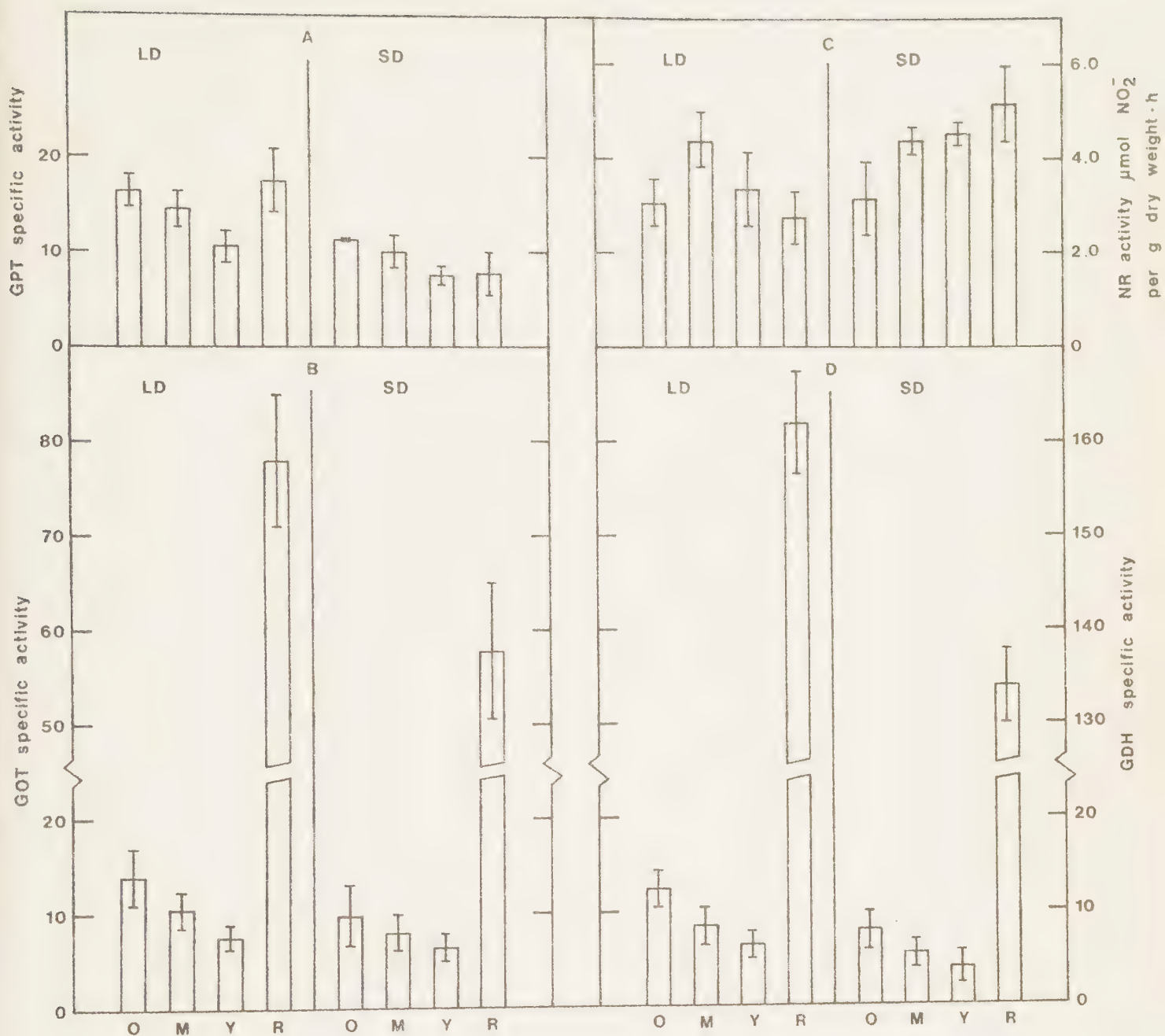


Figure 9. Enzyme activity in different parts of *Spinacia* plants grown under LD and SD conditions.

A: GPT activity in μmol keto acid produced per h and mg protein,

B: GOT activity in μmol keto acid produced per h and mg protein,

C: NR activity in μmol NO<sub>2</sub><sup>-</sup> per g d.wt and h,

D: GDH activity in units per mg protein

Otherwise, data expressed as in figure 1.



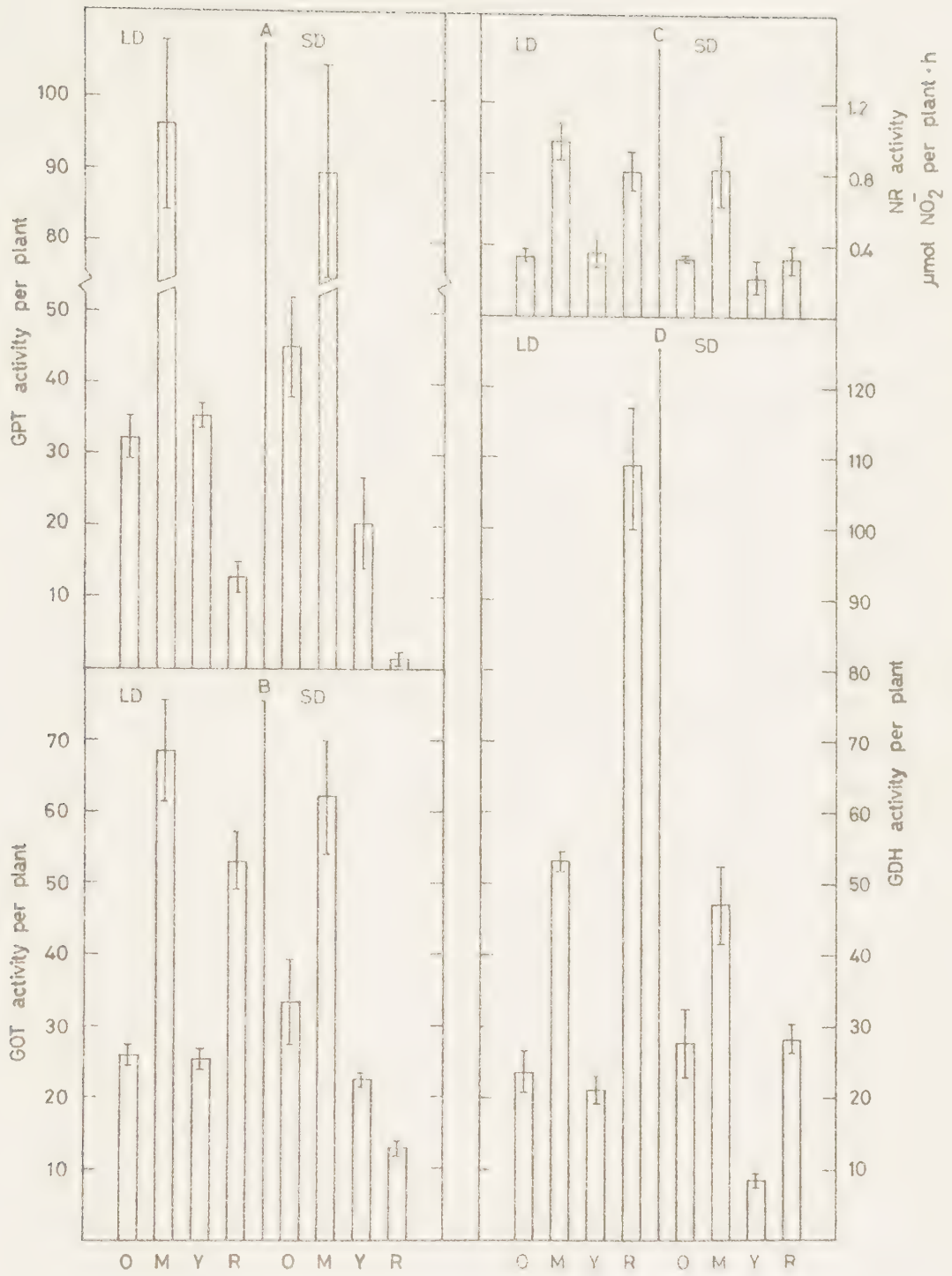


Figure 10. The same as in figure 7, except that enzyme activity is expressed per plant part.



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Investigation of plant morphogenesis and nitrogen metabolism  
during flower induction in *Spinacia oleracea*

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## Introduction

The photoperiodic length affects plant morphogenesis and nitrogen metabolism in *Urtica dioica* and *Spinacia oleracea* (Welanders and Bergkvist 1979). In both species, flowering is induced under long day (LD) conditions. It is not known whether the observed changes are caused directly by the extended photoperiod or indirectly by induction of flowering. In order to deal with this issue, the present investigation is concerned with a study of the course of events occurring in plant morphogenesis and nitrogen metabolism during flower induction. In the present investigation, *Spinacia oleracea* was used as plant material. In order to diminish the effect of photosynthesis, the extended photoperiod was obtained by low intensity light from red fluorescent tubes. Whether a plant is induced to flower or not can be established by following the development of the shoot apex. Therefore, the shoot apices were sectioned with a freeze microtome and examined in a microscope. In addition to several nitrogen fractions, the following enzymes, involved in nitrogen metabolism, were studied: Glutamate dehydrogenase (GDH, E.C.1.4.1.2.), glutamate-oxaloacetate transaminase (GOT, aspartate aminotransferase E.C.2.6.1.1.), glutamate pyruvate transaminase (GPT, alanine aminotransferase E.C.2.6.1.2.), and nitrate reductase (NR, NADH:nitrate oxidoreductase E.C.1.6.6.1.).

Abbreviations: GDH: glutamate dehydrogenase; GOT: glutamate-oxaloacetate transaminase; GPT: glutamate-pyruvate transaminase; NR: nitrate reductase NADH:nicotinamide adenine dinucleotide reduced; TCA: trichloroacetic acid.



### Plant material and cultivation

*Spinacia oleracea* L. (cv. Sperling's Monoppa), a long day rosette plant, was used as plant material. The seeds has been supplied, as a gift, by FDB Frø, forsøgsgartneriet, Toftø, a Danish plant breeding station. This cultivar has been bred in order to exclude all the male plants and provide a more uniform material for flowering experiments. The seeds were sown in soil in 4 litre boxes. After one week, the seedlings were thinned until 30 plants remained in each box, to which 10 g of Weibull's Enpekå Special fertilizer (12% N, 12% P<sub>2</sub>O<sub>5</sub>, 19% K<sub>2</sub>O, and micronutrients) was added. In order to obtain plants of equal size for the experiments, an extensive plant material was prepared, consisting of 16 boxes, each of which contained 30 plants. The plants were distributed to two climate chambers where they were exposed to 10 h photoperiods (SD, non-inductive climate) and kept at a temperature of 22°C. Light was supplied from cool-white fluorescent lamps (F48PG 17/CWC, General Electric) providing an irradiance of 12.8 W·m<sup>-2</sup> in the range 375-715 nm. About four weeks after sowing, the plants in one climate chamber were subjected to 16 h photoperiods (LD, inductive climate). In addition to the 10 h light supply, low-intensity light from red fluorescent tubes (TL15 Philips) providing 1 W·m<sup>-2</sup> was supplied for 6 h. The emission spectrum (W·m<sup>-2</sup>·nm<sup>-1</sup>) of these tubes shows a peak at about 650 nm.

### Sampling and protein extraction

After the plants had been subjected to photoperiods of different length, 10 plants from each photoperiodic regime were harvested 45 minutes after the termination of the dark period and analyzed at days 0, 2, 4, 6, 8, 11, 14, 17, 22 and 28 in the first experiment, and at days 0, 2, 4, 7, 9, 11, 14, 16 and 18 in the second experiment. The plants were selected according to a plant plastochrone index calculated as described by Loewenberg (1970). In the first experiment



use was made of leaves nos 2 plus 3 (one being the oldest leaf) from each plant. In the second experiment, all the leaves were used. The area of each leaf pair from five plants from each photoperiodic regime was measured on every harvest occasion. Laminar area determinations were made from drawings with the aid of a planimeter.

The leaf material was cut and mixed. Four g fresh weight was taken out and extracted as described by Welander (1978). Two separate cultivations were performed in the first experiment, whereas in the second experiment two parallell extractions were accomplished at the same time. In the second experiment, the fresh weight of the shoots, the leaves and the stem plus leaf petioles from each plant was determined. The stem plus leaf petioles and part of the leaf material were freeze-dried and used for dry weight measurements and nitrogen analyses. Part of the protein extract was stored at  $-20^{\circ}\text{C}$  and later used for protein and GDH determinations and in the second experiment also for gel electrophoresis.

#### Histological methods

At every harvest the apices of five plants from each photoperiodic regime were sectioned for morphological studies. Fresh sections of 50-60  $\mu\text{m}$  were cut at a temperature of  $-15^{\circ}\text{C}$  by means of a freeze microtome. The sections were stained in a Zn-I solution. The mixture consisted of 25 g  $\text{ZnCl}_2$  plus 8 g KI dissolved in 8.5 ml water to which 1.5 g I was added. The starch of the plastids was stained black-blue, the proteins yellow to yellow-brown and the cellulose walls violet (J. Elvers). Characterization of the apices was made from photographs and camera lucida drawings.

#### Enzyme determination

All enzyme determinations, except for GDH, were carried out immediately after protein extraction. The activity of NR was measured according to Hageman and Hucklesby (1971) with some modifications as





described by Welander (1978). GDH activity was established according to Schmidt (1963) with some modifications (Welander 1974). In the determinations of GOT and GPT, "optimized UV-test" from Merck (Darmstadt) was used. The activities of GOT and GPT were determined by measuring the rates of NADH consumption by the decrease in absorbance of the test mixtures at 340 nm. The test mixture for GOT had a total volume of 3.2 ml consisting of 0.1 ml enzyme preparation, 90 mM phosphate buffer pH 7.4, 225 mM L-aspartate, 0.20 mM NADH, and 0.6 units/ml of malate dehydrogenase. The test solution was incubated for 5 min at 25°C and the reaction was started by adding 0.05 ml of 768 mM 2-oxoglutarate solution. The same composition was used for GPT, except that the concentration of 2-oxoglutarate in the reaction medium was 18 mM, and aspartate was replaced by 800 mM L-alanine and malate dehydrogenase by lactate dehydrogenase (1.2 units/ml). The activities of GOT, GPT and GDH were expressed as nmol NADH oxidized per min and mg protein, and NR activity as nmol  $\text{NO}_2^-$  produced per h and mg protein.

#### Polyacrylamide gel electrophoresis

The proteins in the extract from the second experiment were separated on 7% polyacrylamide gels prepared in tubes of an inner diameter of 0.9 cm according to the method used by Davies (1964). 200  $\mu\text{l}$  of the protein extract corresponding to 200-400  $\mu\text{g}$  protein was loaded on each gel. The electrode buffer, pH 8.3, was admixed with 2 ml 0.001% (w/v) bromphenol blue solution for detection of the front. The electrophoresis was conducted at 2 mA per tube for 3.5 h. After electrophoresis, the tubes were incubated in a 10% (w/v) trichloroacetic acid (TCA) solution for 30 min. Unspecific protein staining was made in a 1% (w/v) water solution of coomassie brilliant blue diluted with 12.5% (w/v) TCA to 0.1%. The gels were destained by rinsing in a 12.5% (w/v) TCA solution. Densitometer tracings of the gels were obtained as described by Welander (1974).



### Protein and nitrogen determinations

The amount of water soluble proteins was measured by the method of Potty (1969) with small modifications according to Bensadoun and Weinstein (1975). The latter report discloses a procedure for quantitative precipitation of small amounts of protein by the combined use of Na-deoxycholate and TCA. The values obtained for water soluble proteins are divided by a factor 6 and expressed as soluble protein nitrogen. The total protein nitrogen of the dry matter remaining in the residue after TCA-extraction and the TCA-soluble the non-protein nitrogen were determined according to Kjeldahl. For the determination of ammonium and nitrate, nitrogen samples of about 50 mg of freeze-dried material were used. The sample was distilled in 10 ml water and 7.5 ml of 5% (w/v) magnesium oxide and the ammonium nitrogen was collected. Distillation of the same sample was repeated after the addition of 0.3 g Devarda's alloy, thus reducing nitrate to ammonium.

## Results

### Photomorphological changes

The first visible responses of the plants transferred from SD to LD are upright leaf orientation and increased petiole elongation. Figure 1 A and B shows that leaf expansion is more rapid during LD and the final leaf area also increases. The number of leaves produced under non-inductive and inductive conditions is the same and increases linearly with time (data not shown). It is obvious that the increased growth rate is due to the 6 h supplementary light, since plants kept under both LD and SD receive identical high-intensity light during the first 10 h. The more rapid laminar expansion is during LD, the more rapid is the increase in fresh weight for both leaves and stem plus leaf petioles (figure 2 A). The increase in fresh weight does not start until after 7 LD. In stem plus leaf petioles, the increase in fresh weight is accompanied by an increase in dry weight. The increase in fresh weight



of the leaves, on the other hand, results in just a slight increase in dry weight at the end of the experimental period (figure 2 B).

The illustrations (figure 3 A and B) are obtained from camera lucida drawings. Apices from five plants are included in each observation in order to show interindividual variations. Figure 3 A is an account of the changes that the apex undergoes during transition from vegetative to reproductive state, as observed at different times after LD induction. Morphological changes in the apical dome can be seen. At the beginning of the inductive photoperiod, apex is relatively flat and bulges after about seven to nine days. This first visible sign of induction is marked with an arrow in figures 4 and 6-11. Visible flower primordia appear 12-13 days after induction has started, under the conditions prevailing in the experiments of this study. Using 16 h supplementary illumination from incandescent lamps, Zeevart (1971) demonstrated that 7-8 days are needed to induce flower formation. In figure 3 B, the stationary phase of vegetative growth under non-inductive conditions is documented. The characteristic pattern of the relatively flat apical dome prevails until the end of the experimental period. After about three weeks, the apical dome tended to rise in the same way as during LD (data not shown). Some of the plants were kept under non-inductive conditions for several months but the rise of the apical dome resulted only in the formation of sterile flower primordia. These results are consistent with those obtained for *Cannabis sativa* (Heslop-Harrison 1970) and *Spinacia oleracea* (Zeevart 1971) where an "intermediate apex" was demonstrated under non-inductive conditions.

#### Total protein, soluble protein and non-protein nitrogen determinations

The content of protein nitrogen (mg per g d.wt) in leaves from plants exposed to inductive conditions shows a marked upsurge after two LD cycles (figure 4 A) before any visible sign of flower induction is observed. The protein content then decreases to remain on a rather





constant level. An increase of the photoperiod above 16 LD causes a further decrease of the amount of protein. In leaves from vegetative plants, a slow increase in protein content is noted. Maximum increase is reached after 35 days from sowing. The protein content then decreases to a similar level as for induced plants and this level is maintained throughout the experiment. On the other hand, the content of water-soluble protein nitrogen in leaves from plants subjected to LD did not show such a marked upsurge as demonstrated for protein nitrogen (figure 4 B). The changes in the content of soluble protein in leaves from both induced and non-induced plants exhibit the same pattern, although the level is higher for induced plants. After 35 days from sowing, a minimum level of the content of soluble protein is recorded for both induced and non-induced plants. The content of non-protein nitrogen in leaves from plants in LD and SD has a peak after 28 days from sowing (figure 4 C). Induced plants have a second peak in the non-protein content immediately after the first visible sign of induction is recorded.

#### Gel electrophoresis

In the second experiment, gelelectrophoresis of soluble proteins from leaves of photoperiodically induced and non-induced plants was performed. No differences could be observed in the protein pattern between plants in LD and SD either before or after visible flower primordia had been detected. Figure 5 shows the protein pattern of plants in LD and SD analyzed 35 days after sowing. LD plants had by then received 9 inductive cycles and reached the stage when the first visible change of the apical meristem could be noted. On the other hand, similar time-dependent changes in the stained banding pattern could be noted for both induced and non-induced plants. These changes, which are probably due to alterations in the physiological ages of the leaves, concern both the number of protein bands and the height of the peaks (data not shown).



### Nitrate and ammonium determination

The levels of nitrate display a marked increase in stem plus leaf petioles in plants exposed to LD, whereas in SD plants the nitrogen levels are almost constant (figure 6). At the end of the experimental period, twice as much nitrate is found in stem plus leaf petioles in LD as compared with SD. The increased amount of nitrate in stem plus leaf petioles in LD brings about a higher content of nitrate in the leaves (figure 7). Very low and similar levels of ammonium nitrogen were obtained in both leaves and stem plus leaf petioles. No differences attributable to different photoperiods were observed (figures 6 and 7).

### Enzyme determination

The specific activity of GPT temporarily decreases under inductive conditions (figures 8 A and 9 A). This decrease occurs before the first visible change in shoot apex is recorded, thus indicating that the change in the enzyme level is associated with early events related to photoperiodic alterations. When leaf pairs 2 plus 3 are analyzed, the temporary decrease is observed after three LD cycles, whereas when all leaves are analyzed it is observed after two LD cycles. Figure 9 A shows two peaks in the GPT activity in both induced and non-induced plants. The interval between the peaks is eight days. The oscillation of the enzyme activity seems to cease 48 days after sowing. When all leaves are examined (figure 8 A), the oscillations in the GPT activity are less pronounced.

The oscillation in the GOT specific activity in leaves from induced and non-induced plants is very similar to that in the GPT activity and the interval between the peaks is the same (figures 8 B and 9 B). However, the GOT activity does not respond to the photoperiod in the same manner as does GPT. Considering leaf pairs 2 plus 3 (figure 9 B), the GOT activity is very similar for plants in vegetative and inductive climate up to 46 days after sowing. After the induced plants have received 14 LD,



GOT activity decreases to a higher extent than in non-induced plants. Considering all leaves (figure 8 B), differences in the level of GOT activity between induced and non-induced plants are also observed after 14 LD cycles. At this stage, visible flower primordia in the sections of apex are observed, which indicates that the decrease in GOT activity is probably a result of metabolic changes caused by flowering and not due to flower induction.

No such differences in the specific activity of NR are observed as can be related to either induction or evocation of flowering. On the other hand, the activity of NR is lower in leaves from induced plants than in non-induced plants (figures 10 A and 11 A). Oscillation of the enzyme activity is recorded in leaf pairs 2 plus 3 as well as in all leaves, and the interval between the peaks is about seven days. As in the activity of GPT and GOT, the oscillation of the NR activity in leaf pairs 2 plus 3 seems to cease at the end of the experimental period.

Very similar patterns of the specific activity of GDH are obtained in leaves from plants subjected to either LD or SD (figures 10 B and 11 B). In both leaf pairs 2 plus 3 (figure 11 B) and all leaves (figure 10 B), the first differences in GDH activity between induced and non-induced plants are observed after the induced plants have received 16 LD cycles. It is evident that, after the development of flower primordia, GDH activity increases much more markedly than in vegetative plants. The oscillations in the activity of GDH differ from the oscillations found in the other enzymes investigated. The interval between the peaks varies from 4 to 5 days. In leaf pairs 2 plus 3 the oscillations in GDH activity cease much earlier than the oscillations of other enzyme activities.



## Discussion

Photomorphogenetic changes similar to those observed in spinach in this work have previously been demonstrated for beetroot and celery (Soffe et al. 1977) and for spinach (Zeevart 1971) as a result of extended daylength with low intensity incandescent light. In beetroot and celery (Soffe et al. 1977), an increase in leaf area during extended photoperiod also resulted in increased dry weight. Soffe et al. explain the increase in dry weight by the photomorphogenetic increase in leaf area and petiolar growth, improving light interception and permitting a more efficient use of the high intensity light during the rest of the photoperiod. When, in the present investigation, light from red fluorescent tubes was used to extend the photoperiod, the increase in leaf area and leaf fresh weight merely resulted in very small changes in dry weight. Deutch and Rasmussen (1974) have shown that the yield of dry matter is higher in plants subjected to light containing much infrared radiation than in light with little infrared radiation. Incandescent lamps radiate proportionally more in the infrared region than do red fluorescent tubes. This may explain the differences obtained in dry weight.

Variations in the content of nucleic acids and proteins in the leaf during transition from vegetative to reproductive stage have earlier been reported by several scientists but the results are not consistent (Nitsan 1962, Evans 1971, Oota and Umemura 1970). In the present investigation, a temporary increase in the content of total protein nitrogen was observed after two LD cycles in leaves from induced plants. This increase is probably due to the presence of proteins associated with membranes because four LD cycles are





required for obtaining an increase in the content of soluble protein nitrogen. The findings of Kinet (1974) indicate that leaf proteins in *Sinapis alba* are located mainly in the chloroplasts. Kinet suggests that the increase in protein content observed after one LD cycle is a result of the formation of new plastid proteins associated with chloroplast duplication and growth. Sahwney et al. (1976) showed that the content of soluble proteins in leaves, however, not in stems, from *Impatiens balsamina* increases after two inductive LD cycles. However, in the experiments performed by Kinet and Sahwney, the photoperiod was extended with high intensity light, which may influence the photosynthesis.

No consistent changes have been reported in the qualitative pattern of soluble proteins in leaves during floral induction. Oota and Umemura (1970) demonstrated a new protein obtained immediately after floral induction in cotyledons of *Pharbitis nil*, whereas no differences in the protein pattern were found in leaves from *Pharbitis nil* (Sherwood 1970) or in leaves from *Xanthium pennsylvanicum* (Chen et al. 1970). In the present investigation, polyacrylamide gel electrophoretic studies of soluble proteins from leaves of induced and non-induced plants did not reveal any differences in the stained protein pattern.

The content of nitrate in stem plus leaf petioles and in leaves increases during inductive climate (figures 6 and 7). This phenomenon can probably be explained by increased leaf expansion and stem and petiole elongation in LD. During plant growth, cell elongation implicates that the cell wall becomes more plastic, resulting in a decreased turgor pressure, and that more water will move into the cell, resulting in an increase in cell length. The cell can compensate for the dilution by taking up nitrate. The level of ammonium is very low in stem plus leaf petioles and in leaves and is not affected by photoperiodic variations. On the other hand, it is a well known fact that nitrate may accumulate, whereas nitrite and ammonium never do (Hewitt 1970).



The raised amount of nitrate in the leaves during inductive climate does not increase NR activity (figures 10 A and 11 A). In fact, this activity decreases in LD. This indicates that there are different nitrate pools for storage and induction. Different metabolic pools for nitrate have earlier been discussed (Ferrari et al. 1973). Meeker et al. (1974) showed that when leaves of different ages were used, there was found no correlation between nitrate concentration and NR level. Meeker et al. further demonstrated that NR activity increases with leaf development, reaches a maximum with full leaf expansion, and then declines. This may be a possible explanation of the oscillation in NR activity observed in the present investigation (figures 10 A and 11 A). The interval between the two peaks in NR activity recorded for leaf pairs 2 plus 3 coincides with the interval for a plastochrone (figure 1 A and B). This indicates that oscillation in enzyme activity is due to different developmental stages in the leaves. The enzyme pattern for LD and SD is very similar and there is no correlation with the flowering process.

It is well known that red light causes photomorphogenetic responses which are mediated by phytochrome (Borthwick et al. 1961, and Mohr 1962). Many photomorphogenetic responses controlled by phytochrome involve hormones (Black and Vlitos 1971). In the present investigation, GPT is the only enzyme that responds to photoperiodic variation before changes in the apex are observed microscopically (figures 8 A and 9 A). One possible explanation of the temporary decrease in GPT activity during inductive conditions is the effect of a transient increase in the content of endogenous gibberellins (GA). This explanation is supported by the following: Hedley and Stoddart (1971) showed that the activity of GPT is depressed by GA. Radley (1967) found temporary increase in GA activity in spinach after one LD cycle in one experiment and after three LD cycles in another. Zeevart (1971) was able to demonstrate three



gibberellin-like substances, called I, II and III, in spinach in SD. Subjected to LD, substance II increased whereas substance III decreased. Substance I showed an increase after one LD cycle but thereafter decreased. The temporary decrease in GPT activity observed in this investigation after two LD cycles when leaf pairs 2 plus 3 are used and after 4 LD cycles when all leaves are used, coincides in time with the transient increase in GA content observed by Radley and Zeevaart. The control of GA in the flowering process is very uncertain. The oscillations in GPT activity and in GOT activity may be due to different developmental stages of the leaves. As for NR, the interval between the two peaks coincides with the interval for a plastochrone. This may explain why the oscillations seem to cease when only two leaf pairs are analyzed. Correlation between enzyme activity and laminar area has been demonstrated by Steer (1971). The observed changes in the activity of GOT and GDH (figures 8 B, 9 B, 10 B and 11 B) in LD and SD occur after visible flower primordia in the sections of apex have been observed. From these results, it can be concluded that at least GOT, GDH and NR are not involved in the flower induction process.





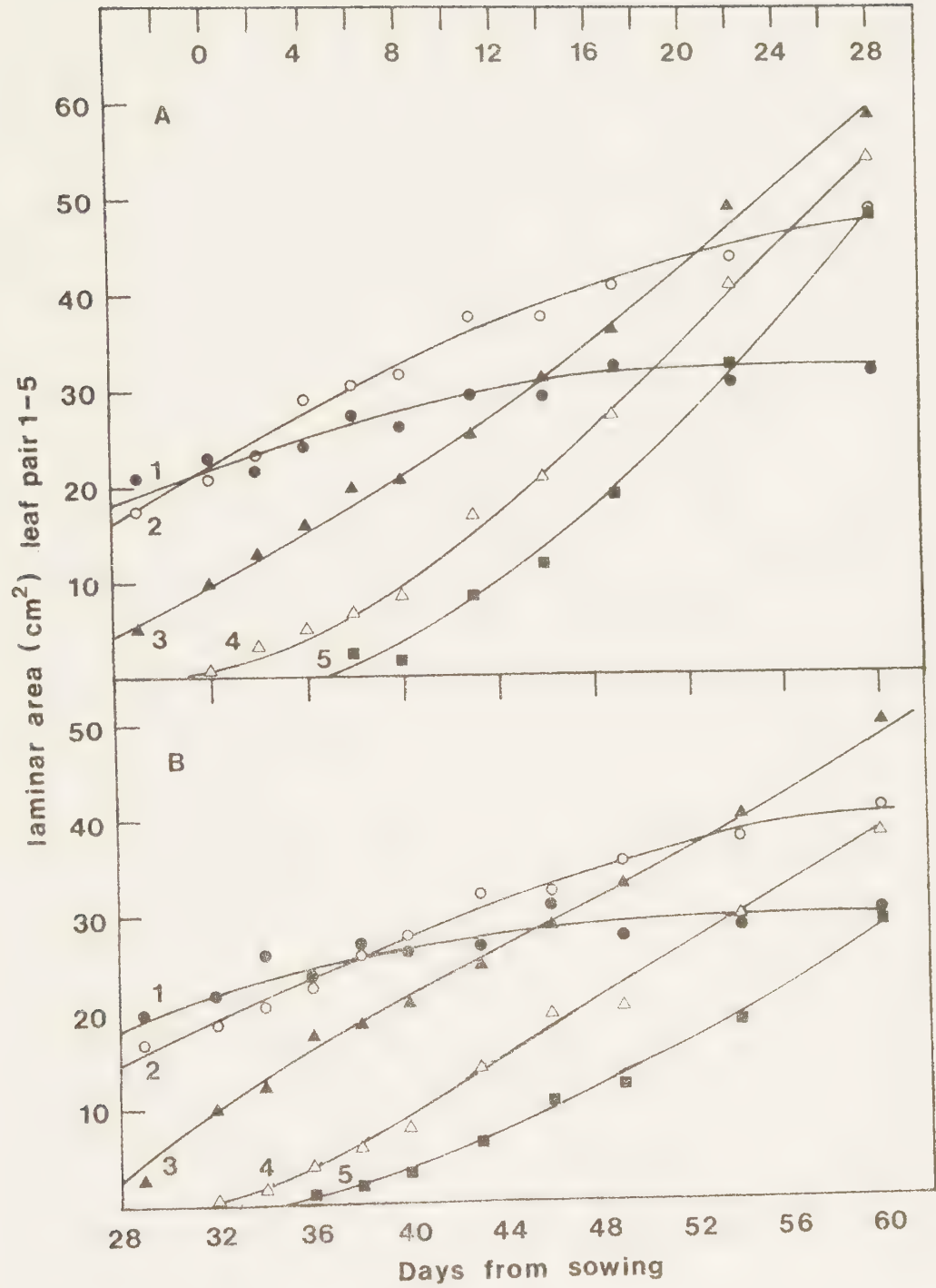


Figure 1. Growth in laminar area (cm<sup>2</sup>) of leaf pairs 1-5 with time. Leaves from plants in LD (A) and in SD (B). The abscissa in A indicates the numbers of inductive cycles. Each point represents a mean value of 5 plants. Leaf pairs 1 (●), 2 (○), 3 (▲), 4 (△), and 5 (■).



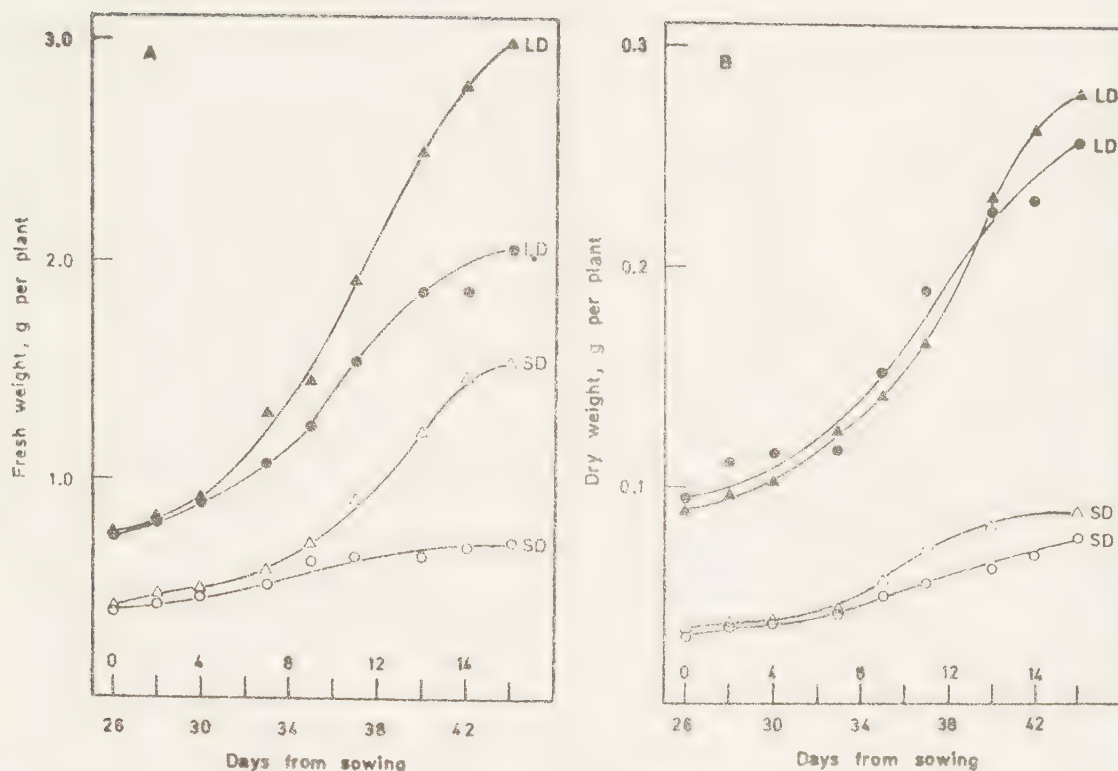


Figure 2 A. Changes in the amount of fresh weight with time in leaves (▲) and stem plus leaf petioles (●) from plants in LD, and leaves (Δ) and stem plus leaf petioles (○) from plants in SD. Each value represents a mean of ten plants. The numerals 0-16 above the abscissa indicate the numbers of inductive cycles.

B. Changes in the amount of dry weight with time for plants in LD and SD. Symbols as in A.





Figure 3 illustrates camera lucida drawings of apices from plants after a certain number of inductive cycles (A), and apices from plants kept under vegetative conditions (B). Numerals in brackets state days from sowing. Total laminar area (cm<sup>2</sup>) is given under each apex. Five apices from each observation are included in order to show individual variations.



B. Vegetative plants



Figure 5 illustrates camera lucida drawings of apices from plants after a certain number of inductive cycles (A), and apices from plants kept under vegetative conditions (B). Numerals in brackets state days from sowing. Total laminar area ( $\text{cm}^2$ ) is given under each apex. Five apices from each observation are included in order to show individual variations.





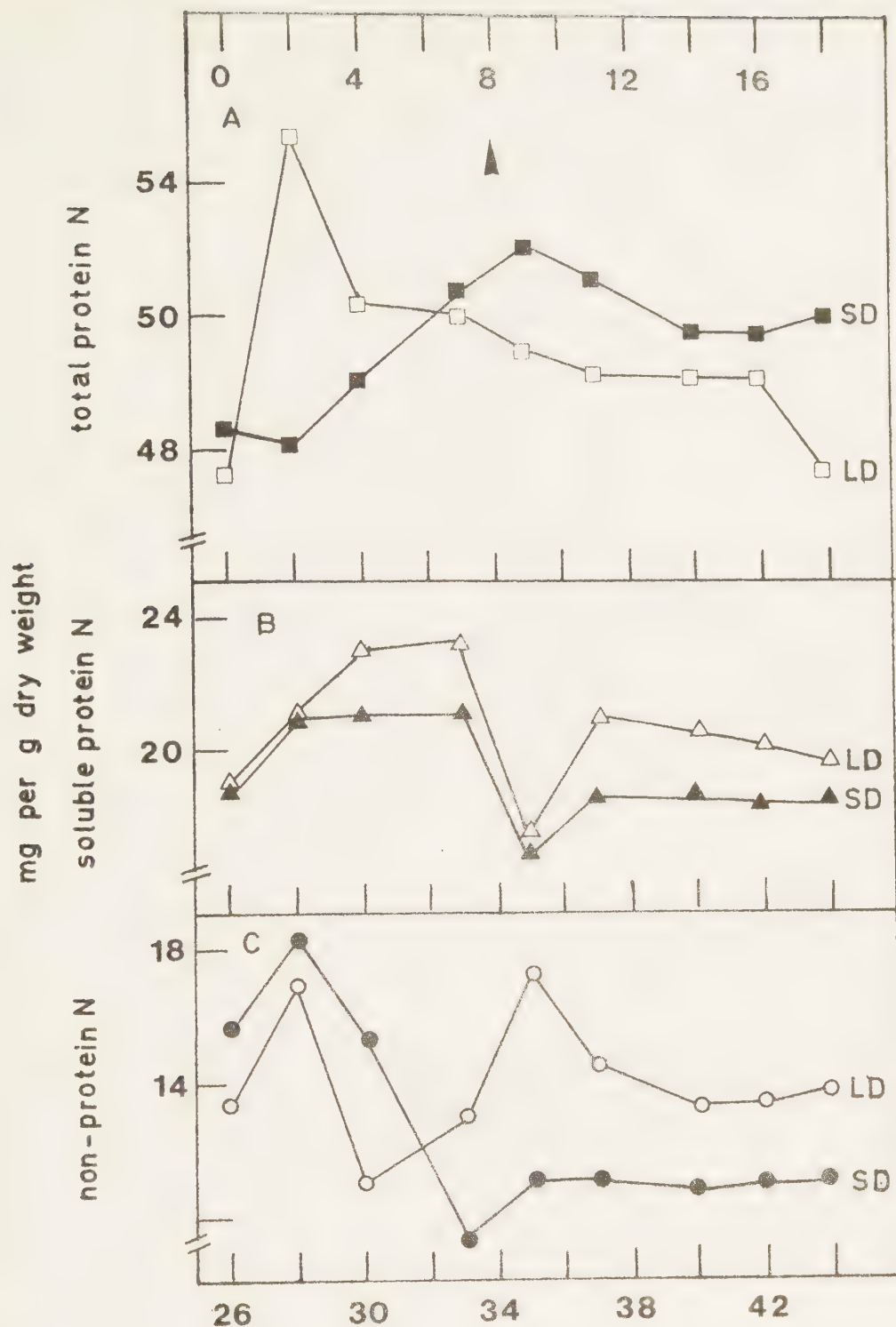


Figure 4. The content of total protein nitrogen (A), soluble protein nitrogen (B) and non-protein nitrogen (C) expressed as mg per gram dry weight in leaves from plants in LD and SD. The numerals given on the abscissa in figure A denote the numbers of inductive cycles. ▲ = the first visible sign of flower induction in sections of the apical meristem.





Figure 5. Densitometer tracings of water soluble proteins after polyacrylamide gel electrophoresis. Leaves from induced (A) and non-induced (B) plants. The samples are taken when plants in LD have received 9 inductive cycles, a stage when changes in the apical meristem can be observed microscopically.



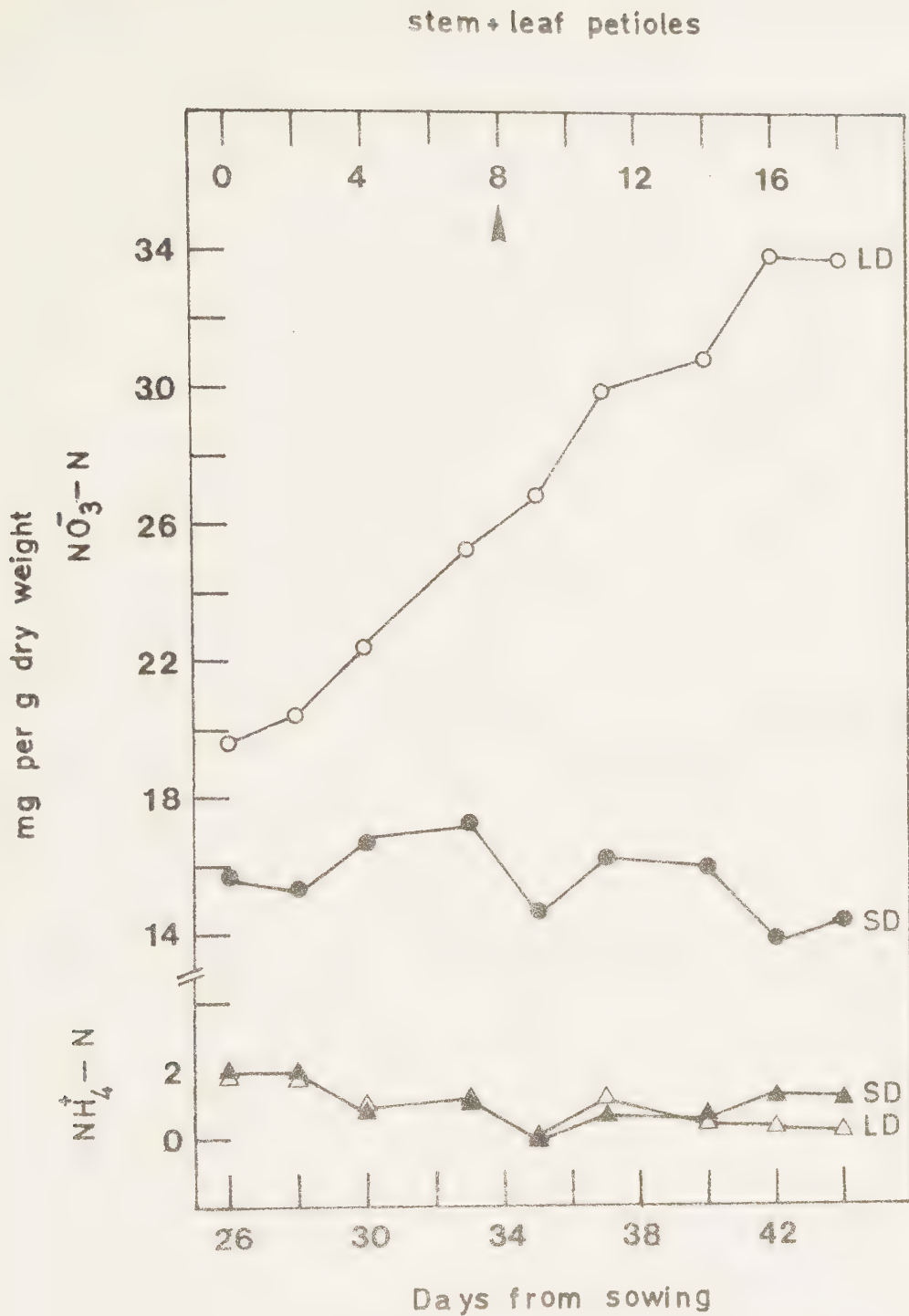


Figure 6. The content of nitrate and ammonium nitrogen expressed as mg per gram dry weight in stem plus leaf petioles from plants subjected to LD and SD.  $\text{NO}_3^- - \text{N}$ , LD (O),  $\text{NO}_3^- - \text{N}$ , SD (●),  $\text{NH}_4^+ - \text{N}$ , LD (Δ), and  $\text{NH}_4^+ - \text{N}$ , SD (▲). The numerals 0-16 denote the numbers of inductive cycles in LD. The first visible sign of flower induction in sections of the apical meristem (▲).





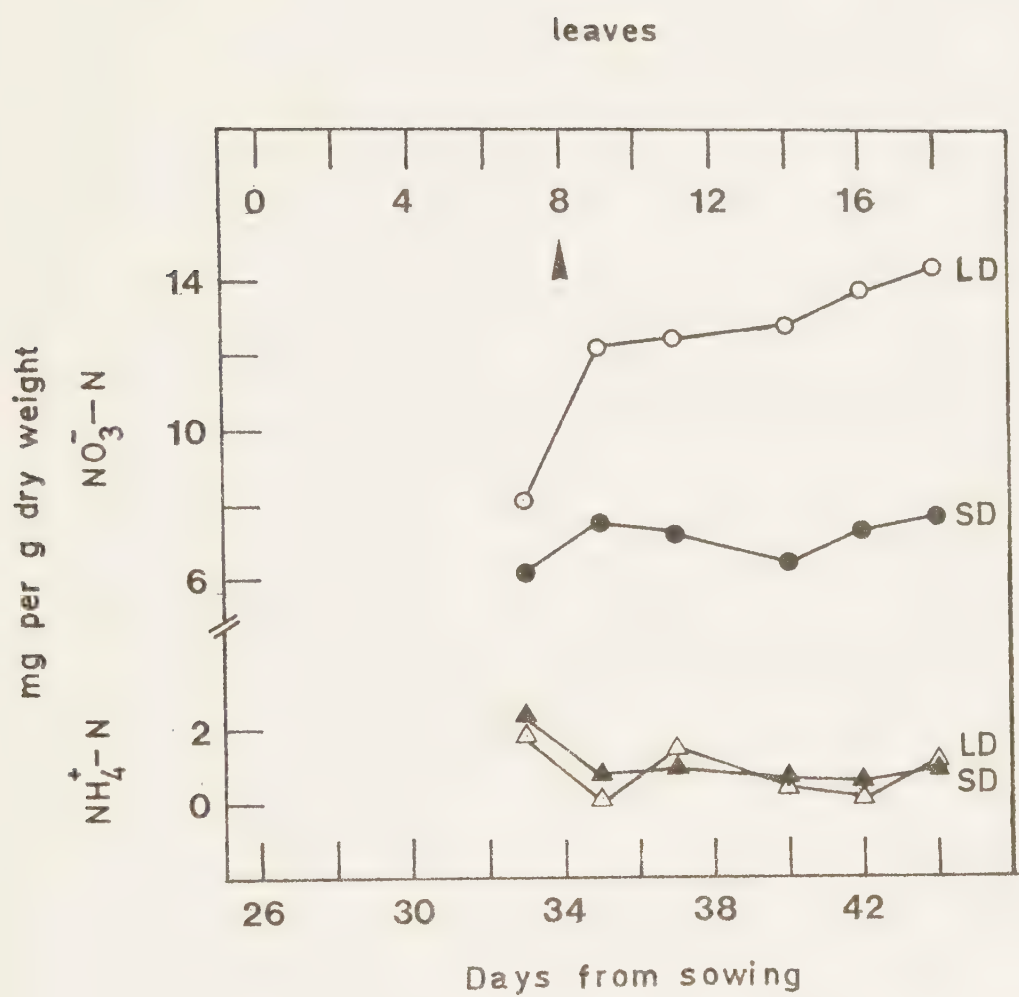


Figure 7. The content of nitrate and ammonium nitrogen expressed as mg per gram dry weight in leaves from plants subjected to LD and SD. Symbols as in figure 6.



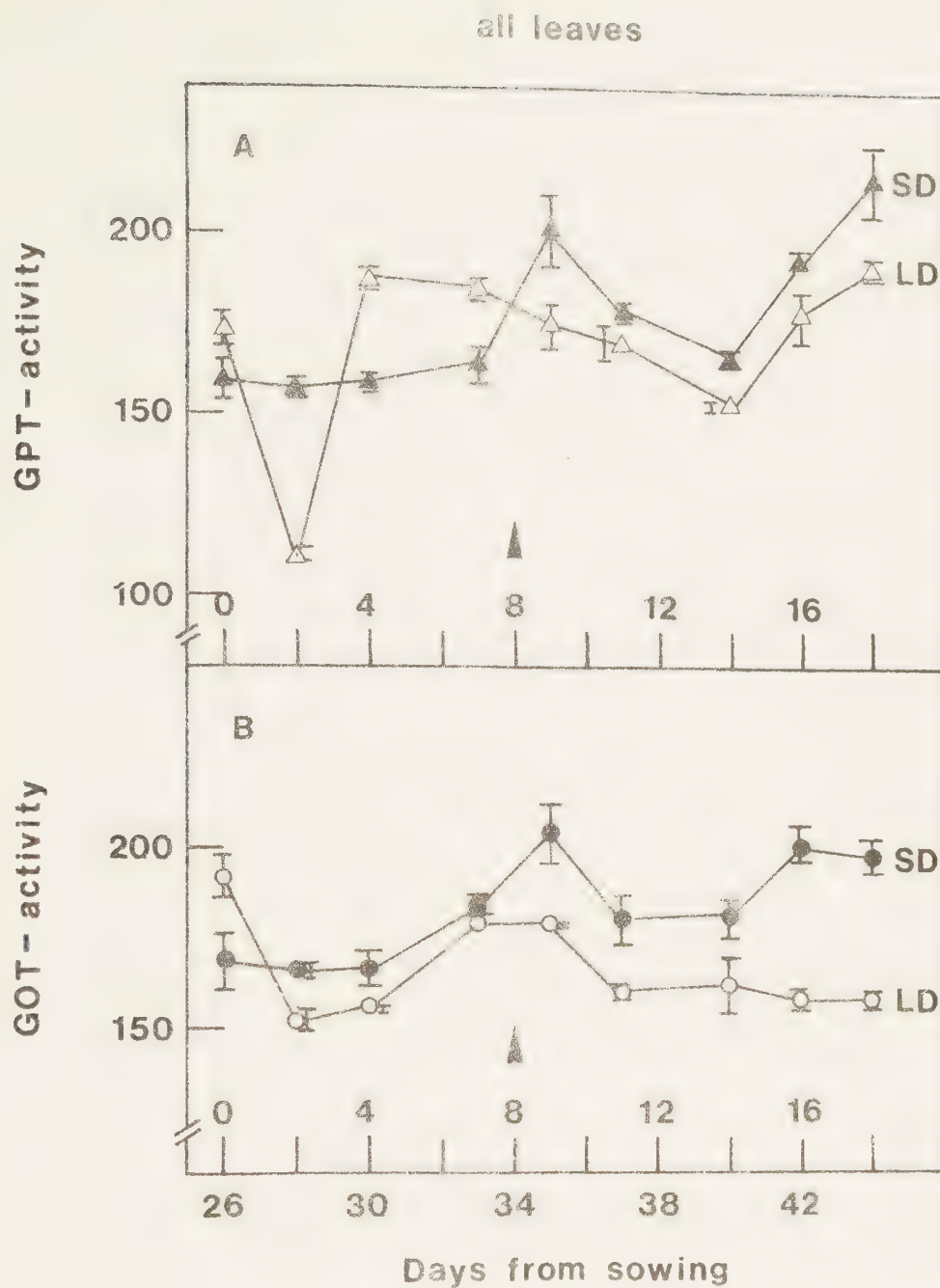


Figure 8 A. Specific activity of alanine aminotransferase (GPT) in all leaves from plants subjected to LD (△) and SD (▲). B. Specific activity of aspartate aminotransferase (GOT) in all leaves from plants subjected to LD (○) and SD (●). The activity of GOT and GPT is expressed as nmol NADH oxidized per min and mg protein. Each plot represents a mean obtained from two parallel extractions and the vertical bars denote the difference between the two extractions. The numerals 0-16 denote the numbers of inductive cycles in LD. ▲ = the first visible sign of flower induction in sections of the apical meristem.



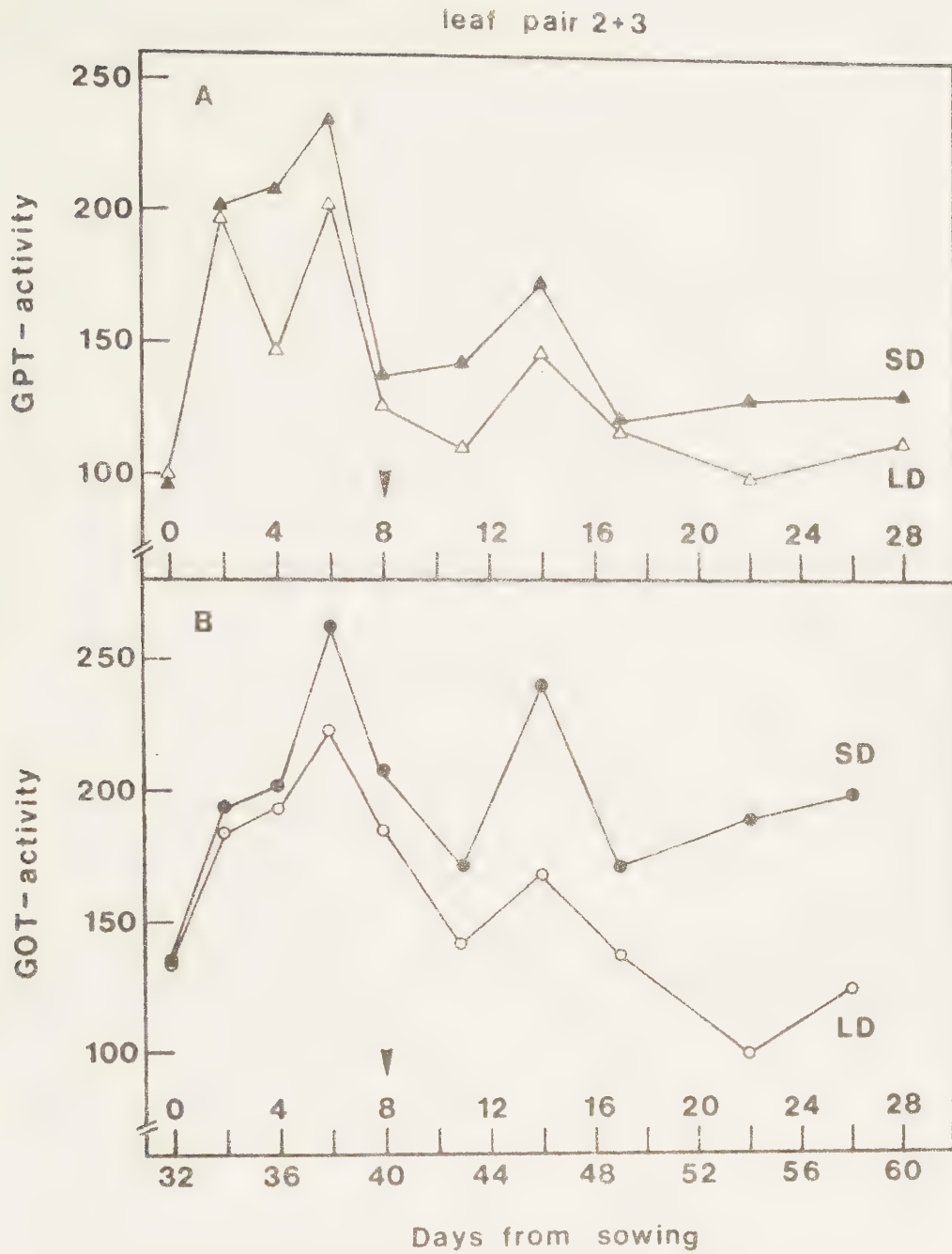


Figure 9 A. Specific activity of alanine aminotransferase (GPT) in leaf pairs 2 plus 3 from plants subjected to LD (Δ) and SD (▲). B. Specific activity of aspartate aminotransferase (GOT) in leaf pairs 2 plus 3 from plants subjected to LD (○) and SD (●). The values in A and B correspond to cultivation I. Data from cultivation II show the same pattern. Otherwise, data expressed as in figure 8.



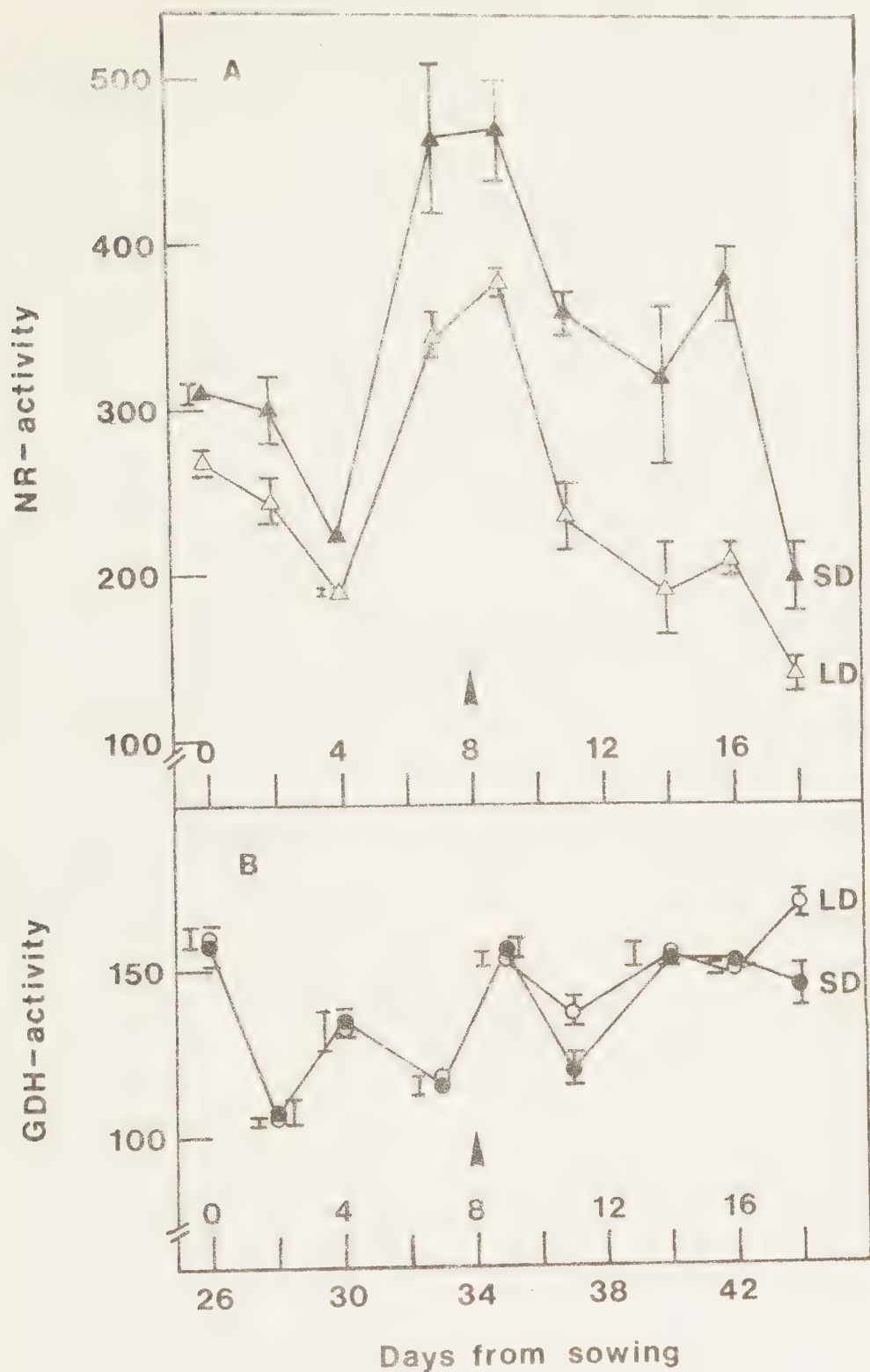


Figure 10 A. Specific activity of nitrate reductase (NR) in all leaves from plants subjected to LD ( $\Delta$ ) and SD ( $\blacktriangle$ ). The NR activity is expressed as  $\text{nmol NO}_2^-$  per h and mg protein. B. Specific activity of glutamate dehydrogenase (GDH) in all leaves from plants subjected to LD (O) and SD ( $\bullet$ ). GDH activity is expressed as  $\mu\text{mol NADH}$  oxidized per min and mg protein. Otherwise, data expressed as in figure 8.





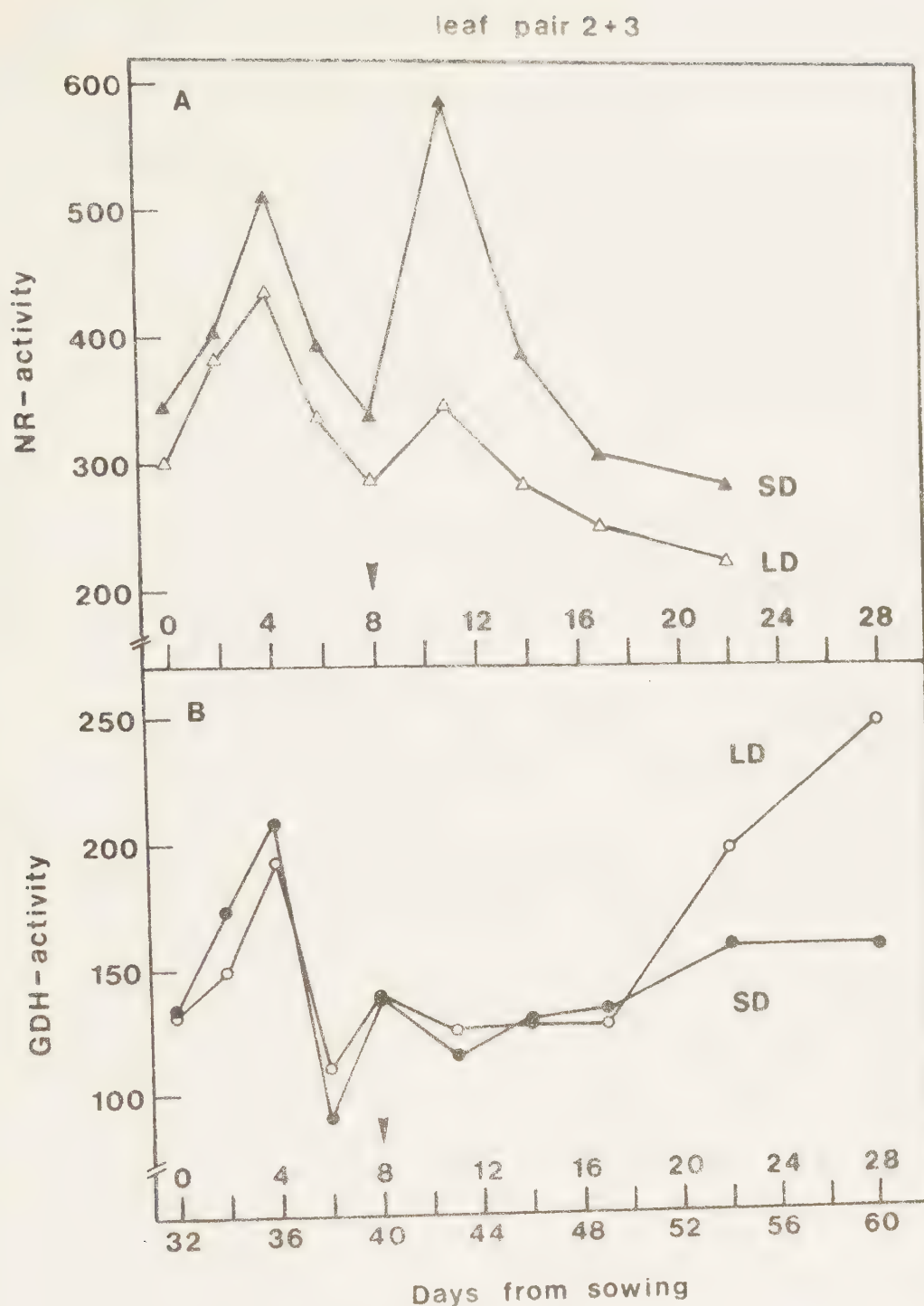


Figure 11 A. Specific activity of nitrate reductase (NR) in leaf pairs 2 plus 3 from plants subjected to LD ( $\Delta$ ) and SD ( $\blacktriangle$ ). NR activity is expressed as  $\mu\text{mol NO}_2^-$  per h and mg protein. B. Specific activity of glutamate dehydrogenase (GDH) in leaf pairs 2 plus 3 from plants subjected to LD (O) and SD ( $\bullet$ ). GDH activity is expressed as nmol NADH oxidized per min and mg protein. The values in A and B correspond to cultivation I. Data from cultivation II show the same pattern. Otherwise, data expressed as in figure 8.



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